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The Role of Connective Tissue Growth Factor (CTGF) in Fibrosis Associated With Intestinal Neuroendocrine Tumors

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

By

Michael David Shapiro

2005

ABSTRACT

THE ROLE OF CONNECTIVE TISSUE GROWTH FACTOR (CTGF) IN FIBROSIS ASSOCIATED WITH INTESTINAL NEUROENDOCRINE TUMORS.

Michael D. Shapiro, Mark Kidd, and Irvin M. Modlin. Section of Surgical Gastroenterology, Department of Surgery, Yale University, School of Medicine, New Haven, CT.

Carcinoid tumors of the small bowel often present with fibrosis in the peritumoral tissues, distant in the heart or lungs, and locally in the peritoneal cavity. The mechanism of the fibroblastic lesions in patients with small bowel carcinoids is unclear and their timely diagnosis impossible. There exists no test to determine the risk of fibrosis, detect its presence, or monitor its progression once discovered. Furthermore, no current therapy protects against such fibrosis. We have proposed that CTGF, a mediator of the profibrotic activities of TGFβ1 (a known regulator of fibrosis) is directly involved in the genesis of ileal carcinoid-related fibrosis. The aim of this study was to assess the potential correlation of serum and tissue CTGF with the diagnosis of carcinoid-related fibrosis. Serum and tissue samples from patients with GI carcinoids, other GI and extra-GI malignancies, and control patients were collected prospectively. A GI carcinoid tissue microarray (TMA) was stained immunohistochemically with anti-CTGF, semi-quantitatively measured, and analyzed for correlation with clinical fibrosis. Significantly higher serum CTGF levels were found in patients with ileal carcinoids than in patients with gastric ECL cell carcinoids (the latter of which are not associated with fibrosis) and control patients. Our results demonstrated that CTGF protein is over-expressed in small bowel carcinoid tumors associated with fibrosis and that the secreted protein is stable and detectable in patient serum. The correlation of CTGF with TGFβ1 suggests that CTGF is a co-secreted fibrotic factor. Since the relationship of CTGF to fibrosis is well defined, this cytokine may be involved in the genesis of ileal carcinoid-related fibrosis. The detection of elevated levels may provide a diagnostic opportunity to predict fibrosis and pre-empt its local and systemic complications. Furthermore, CTGF may represent a therapeutic target for management of fibrosis-related complications in patients with carcinoid tumors.

ACKNOWLEDGEMENTS

"Those having torches will pass them on to one another"

-Plato, The Republic

Inscribed above the entrance to the Sterling Hall of Medicine

I would like to dedicate this thesis to the following:

To **Siegfried Oberndorfer**, for igniting the torch;

To **Professor Irvin Modlin**, for passing the torch; and

To **Milton Charles Winternitz**, founder of the "Yale System" of medical education, for helping me appreciate the importance of passing the torch onto others.

I am particularly obliged to **Dr. Mark Kidd** for his tremendous support, patience, insight, and outstanding guidance. I would also like to thank Dr. John Forrest and Donna Carranzo in the Office of Student Research at the Yale School of Medicine for their assistance with sponsoring this project. Research support was generously provided by the NIH Cancer Education Grant/Yale Cancer Center Medical Student Research Fellowship, NIH-NHLBI Medical Student Research Fellowship, and the Richard Hirshfield Medical Student Research Fellowship.

"We need a system, and we shall surely have it, which will produce not only surgeons, but surgeons of the highest type, men who will stimulate the first youths of our country to study surgery and to devote their energy and their lives to raising the standard of surgical science...[residents] are expected in addition to their ward and operating room duties, to prosecute original investigations and to keep in close touch with the work in surgical pathology, bacteriology, and, as far as possible, physiology"

-William S. Halsted (1852-1922)

Delivered at Yale University, 1904

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INTRODUCTION

Carcinoid tumors are enigmatic, slow-growing neuroendocrine neoplasms derived from the serotonin-producing enterochromaffin (EC) cell and are most commonly associated with the gut and broncho-pulmonary system. The secretion of the tumor's vasoactive substances, particularly serotonin, can manifest systemically with paroxysmal flushing, diarrhea, cardiac valve abnormalities, increased skin pigmentation and bronchospasm—the classic hallmarks of the carcinoid syndrome[1]. In many instances, however, they are identified at surgery for unexplained bowel obstruction or during exploration of the small bowel in search of a primary tumor once distant metastases have been detected.

Despite medical and therapeutic advances that have alleviated symptoms and prolonged life (particularly somatostatin [SST] receptor targeted pharmacotherapy)[2], a substantial subset of patients with midgut carcinoids develops mesenteric and intestinal carcinoid fibrosis and/or carcinoid heart disease. Both of these conditions reflect a connective tissue disorder whose etiology, biology and therapy are unknown[3]. Tumor growth results in a marked fibrogenic response with the development of peritoneal and mesenteric fibrosis (16-48%)[4], the management of which has become a dominant clinical issue. In contrast, gastric carcinoids, derived from the histamine-secreting enterochromaffin-like (ECL) cell of the stomach, are not associated with the development of fibrosis.

The mechanism of the fibroblastic lesions in small bowel carcinoid patients is unclear and their timely diagnosis impossible unless fortuitous intestinal obstruction leads to early surgery. As patients survive longer and are relatively symptom-free because of SST analogue therapy, the issue of carcinoid-engendered fibrosis has emerged as the most critical and

completely unanswered question in the management of patients with these tumors[4]. There is no understanding of the mechanistic basis of fibrosis, no method to detect it before it causes either bowel obstruction or cardiac valve dysfunction, and no treatment to control carcinoid-related fibrogenesis. The development and consequences of fibrosis in patients with carcinoid tumors has thus become a critical quality-of-life issue, since symptom regulation and isotopic and embolic control of tumor growth have increased life expectancy. The advantageous outcomes of such therapy are negatively impacted by the consequences of this uncontrollable neoplastic-driven desmoplastic response. While fibrosis is a ubiquitous process involved in many different diseases[5], it is of particular importance in carcinoid patients, as survival and quality-of-life issues have now become increasingly influenced by the development of peritoneal and/or peripheral (cardiac) fibrosis[4]. Serotonin has been implicated as the mediator of the fibrosis associated with midgut carcinoids; however, no consistent relationship between carcinoid-induced mesenteric fibrosis and elevated blood or tumor levels of serotonin or bradykinin is evident[6]. The responsible factor is thus unknown.

Connective tissue growth factor (CTGF) is a novel, cysteine-rich peptide[7], belonging to a family of immediate-early genes that are especially needed for the coordination of complex biologic processes such as differentiation and tissue repair[7]. CTGF functions as a downstream mediator of transforming growth factor beta 1 (TGFβ1 – a known regulator of fibrosis) action in fibroblastic cells, and mediates some of the profibrotic activities of TGFβ1. Recent studies suggest that TGFβ1 leads to the induction of CTGF, which acts in concert with TGFβ1 to drive the overproduction of collagen, a critical determinant in fibrosis[8]. TGFβ1 has been identified in small bowel carcinoids and in cardiac autopsy material[9]. Whereas TGFβ1 plays an essential role in the initiation of fibrosis, it is the

persistent, TGFβ1-independent CTGF expression characteristic of fibrotic lesions that bypasses the controls that normally suppress the wound healing response, resulting in sustained, chronic, pathological fibrosis[10]. Indeed, CTGF shows an expression pattern that correlates with the severity of fibrosis; that is, CTGF expression is abundant in fibrotic lesions, even in the absence of markedly elevated amounts of TGFβ1 ligand[11].

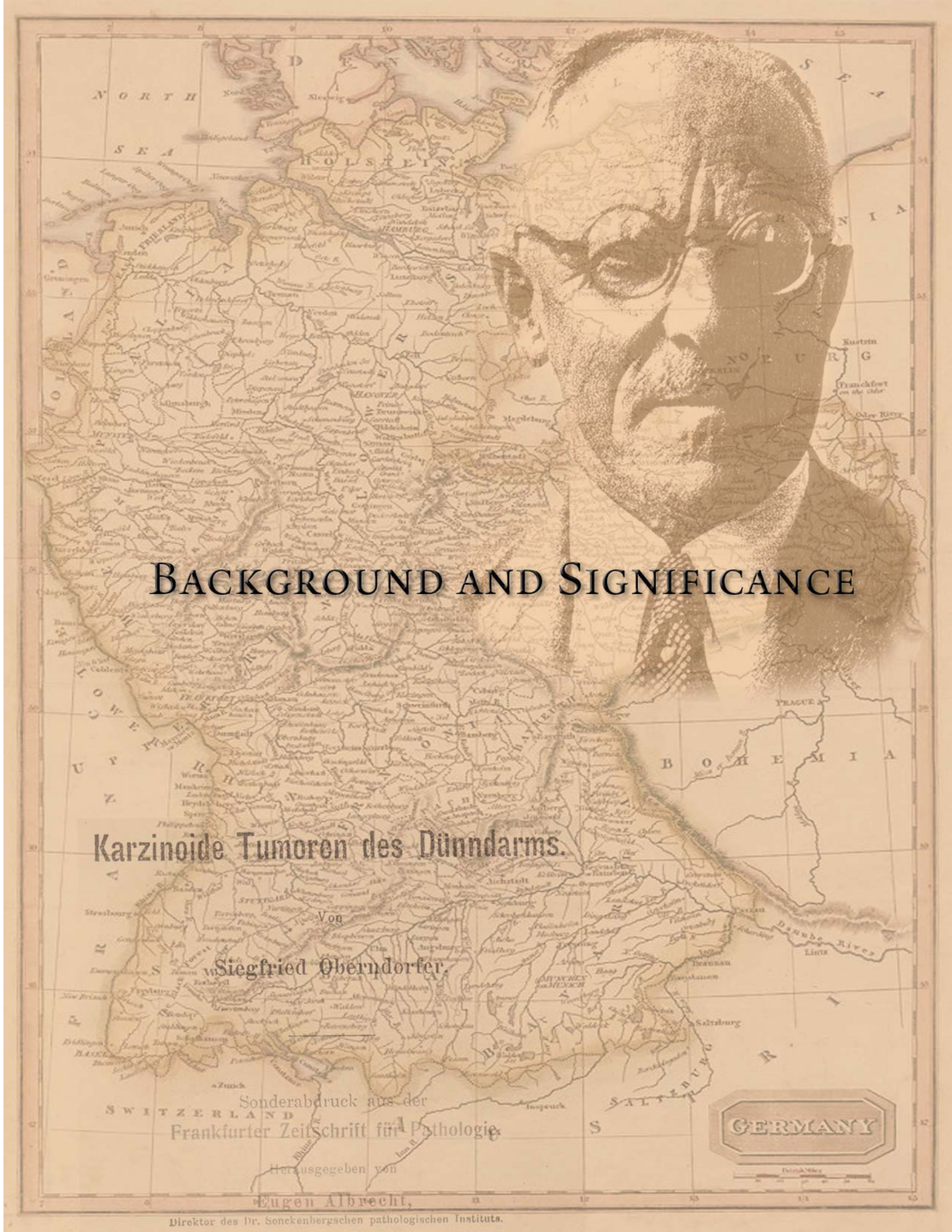
Because the association of small bowel carcinoid tumors with fibrosis and the profibrotic effects of CTGF have been well-established, it is therefore plausible that this bioactive peptide may be intrinsically involved in the regulation of carcinoid-related fibrosis. To acquire preliminary data, CTGF mRNA levels in tumor tissue from patients with carcinoids were examined and compared to levels found in non-tumor tissue from adjacent sites in the same patients, when such tissue was available. Semi-quantitative polymerase chain reaction (PCR) analysis demonstrated that significantly more carcinoid tumor tissue specimens express CTGF message (16/19; 84%) compared to normal tissue (4/9; 44%) ($p<0.05$). Of note, the three carcinoid tumors negative for CTGF message production were all gastric in origin. Subsequent serum analysis of CTGF and TGFβ1 levels in patients with carcinoid tumors, normals, and in patients with other diseases requiring surgery (e.g. ulcerative colitis, colorectal cancer, hernia repair) demonstrated that both CTGF and TGFβ1 levels in patients with ileal carcinoids were significantly elevated ($p<0.02$) compared to patients with other tumors. Furthermore, an examination of these two parameters revealed a strong positive correlation between the two ($R^2=0.81$, $p=0.0004$), demonstrating that CTGF and TGFβ1 are indeed co-secreted. CTGF levels correlated positively with serum Chromogranin A (CgA) levels (a marker of carcinoid tumor size and disease activity) ($p=0.02$), suggesting that CTGF may not only be a marker for fibrosis but a marker specific

to small bowel EC carcinoid tumors as well. Immunohistochemical examination of a gastrointestinal carcinoid tissue microarray (TMA), comprised of tumor samples from thirty-six patients with carcinoids of the small bowel identified at Yale University over the last twenty years (and presently the only carcinoid microarray in the United States), demonstrated that 80% of carcinoids were both TGFβ1- and CTGF-positive, with a correlation between staining of r=0.57 (p<0.0001).

Based on this preliminary data, *I hypothesize that CTGF is over-expressed in small bowel EC carcinoid tumors and is directly involved in the genesis of ileal carcinoid-related fibrosis*. My overall aim is to understand the role of CTGF in the mechanism of carcinoid fibrosis and whether measuring serum levels of CTGF could facilitate the detection of such fibrosis, with the ultimate goal of identifying a novel molecular target for therapeutic intervention in fibrotic disease associated with carcinoid tumors. Furthermore, it is important to understand why carcinoids derived from the enterochromaffin (EC) cell in the small bowel generate a fibrotic response while carcinoid tumors elsewhere in the gastrointestinal tract do not. For instance, it is well-known that gastric carcinoids derived from the gastric ECL cell, irrespective of their metastatic potential, are never associated with fibrosis[12] and therefore constitute an ideal control group. The preliminary data demonstrated that the tissue and serum CTGF levels in patients with gastric ECL cell carcinoids were low (<10 ng/ml). Since carcinoid tumors in different parts of the gastrointestinal tract originate from different endocrine cells and only small bowel (EC cell) carcinoids are associated with fibrosis, determining whether CTGF is the secretory product of this tumor responsible for engendering fibrosis, defining the mechanism by which it causes fibrosis, and identifying a method by which the agent can be identified in tissue and serum could thus facilitate the early

detection or prediction of the likelihood of fibrosis. Since not all small bowel carcinoids are associated with the same rate or degree of fibrosis, this could enable the determination of important scientific as well as clinical information as to the risk of fibrosis in individual patients. Identifying serum factors will enable the identification and monitoring of patients liable to develop fibrosis.

No current strategies exist to treat fibrosis associated with small bowel carcinoids except surgical intervention[13,14]. As such, the identification of a fibrotic mediator of small bowel carcinoid-related fibrosis may allow for the identification of a specific molecular target necessary to identify and develop novel therapeutic probes designed to obviate fibrosis.



Background and Significance

Karzinoide Tumoren des Dünndarms.

Von

Siegfried Oberndorfer.

Sonderabdruck aus der
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Herausgegeben von
Eugen Albrecht,
Direktor des Dr. Senckenbergschen pathologischen Instituts.

Carcinoid Tumors: The Evolution of Our Understanding

Overview

Carcinoid tumors of the small bowel (midgut) are the most common (42%) neuroendocrine tumor (NET) of the gastrointestinal (GI) tract. Derived from the serotonin-secreting enterochromaffin (EC) cell, small bowel carcinoid tumors are histologically as diverse as the spectrum of substances they secrete. Densely packed with neurosecretory granules containing hormones and biogenic amines, they can range from indolent, unrecognized entities to highly active, metastatic secretory tumors[15]. They are generally advanced at diagnosis, and in most instances are only detected at surgery when hepatic metastases have resulted in overt symptomatology or emergently for unexplained bowel obstruction or bleeding. For the most part they tend to be associated with the gastrointestinal tract and the bronchopulmonary system, though there is a substantial number of other sites where these lesions may arise, including the esophagus, Meckel's diverticulum, liver, pancreas, and biliary tract; within the pelvic and oto-laryngeal organs; as well as within the breast[16].

Preface

The historical data in this section pertaining to Dr. Siegfried Oberndorfer is entirely novel and was collected over the course of a year from several sources, including meeting personally with Dr. Oberndorfer's grandson in Germany in June 2004, telephone conversations with Oberndorfer's daughter in Oslo in August 2004, and personal

correspondence with great-grandchildren in Medellín, Columbia and faculty members from the Department of Pathology at the University of Istanbul. Furthermore, I translated an autobiography that Dr. Oberndorfer had written in German a year before his death but never published, and the biographical information presented in the thesis represents a distillation of his own words. The German text was translated manually into English using free online translation software (e.g. AltaVista Babel Fish Translation tool; http://babelfish.altavista.com).

The data detailing the evolution of the carcinoid concept, particularly the descriptions of the earlier (19th century) observations (e.g. those of Langhans, Lubarsch, Notthafft, Nicolas, etc.), were obtained by translating the text of the original German or French articles, which I procured either in the Yale Historical Library or from the private collection of Dr. Castrillón-Oberndorfer and translated manually as described above.

I designed all of the illustrations in this section. I obtained the collage elements from their original sources in the Yale Historical Library or from the personal collection of Walter Castrillón-Oberndorfer in Germany and designed each collage myself using Adobe Photoshop software. Likewise, the schematic cartoons illustrating the proposed mechanisms underlying the hypothesis were conceived of and prepared solely by me using Corel Draw software. Photographs of gross pathological specimens and endoscopy findings were obtained with permission from Dr. Modlin's archived collection. Certain collages have been published recently (e.g. figures 1, 2, 5, and 7 appear in *Modlin IM, Shapiro MD, Kidd M. Siegfried Oberndorfer—Origins and Perspectives of Carcinoid Tumors. Hum Pathol 2004; 35(12):1440-51*).

Historical Background of Carcinoid Tumors

With ever-increasing recognition and understanding of carcinoid tumors, little is known about the lives of the men who first defined the tumor, described its distinct histology and cell type, and the delineated the clinical hallmarks of the disease. Siegfried Oberndorfer (1876-1944), a German pathologist at the University of Munich, first described the idiosyncratic nature of these tumors and coined the term "carcinoid" in 1907. Such lesions had actually been observed and documented earlier, however, by a number of physicians during the nineteenth century, including Theodor Langhans in 1867[17], Otto Lubarsch in 1888[18], and William B. Ransom in 1890[19].

Langhans, who at one time worked under F. Recklinghausen (1833-1910), was the first to describe a carcinoid tumor that was found at autopsy in a fifty-year-old woman who had died of tuberculosis[17]. Langhans noticed a firm, mushroom-shaped submucosal tumor that projected into the lumen of the small intestine, the borders of which were very sharp without any evidence of peri-tumoral invasion (*Figure 1*). He noted how the tumor cells resembled poorly-differentiated glandular tissue and were arranged in nests with a rich, thick fibrous stroma. His 1867 manuscript detailed the histological features of the tumor, though he never commented on the clinical and growth behaviors of this hitherto undocumented neoplasm. The carcinoid tumor had been discovered, though twenty-one years would pass before this obscure and unexplained entity was reintroduced into the medical literature by Otto Lubarsch.

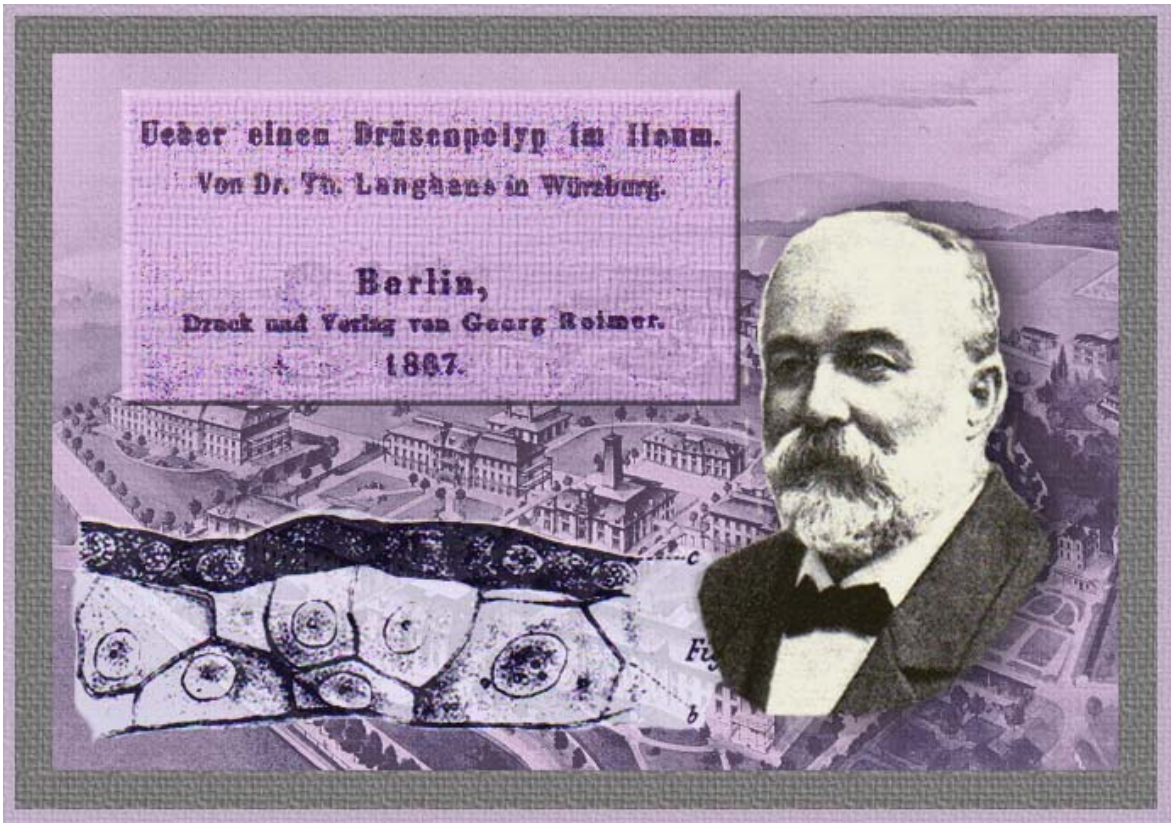


Figure 1*: Theodor Langhans (1839-1915) (bottom right) was born in Usingen, Germany and studied medicine in the universities of Heidelberg, Berlin, and Göttingen. He spent his early career as a pathologist in Würzburg, where in 1862 he was made assistant to von Recklinghausen. Five years later, Langhans published the first report of a carcinoid tumor that was found at autopsy in the small intestine of a 50-year-old woman (frontispiece, top left), though this was merely a histological description of the tumor without any mention of its associated clinical behavior. Langhans later studied the structure of the placenta in Marburg, where he also made significant contributions to the pathology of nephritis, calling attention to the giant cell, which came to be known as the Langhans cell (bottom left). He spent the majority of his career (40 years) at the University of Bern (background), working with the likes of T. Kocher (1841-1917) and H. Sahli (1856-1933), and made major contributions to the understanding of the pathological anatomy of goiter and cretinism, renal infections, blood pigments, and the placenta.*

Lubarsch (1860-1933) of Berlin (*Figure 2*) conducted most of his early research in various Institutes of Pathology throughout Switzerland. In 1888, at the age of 28, Lubarsch described two cases of ileal tumors discovered at autopsy[18]. In one case (49-year-old male), the ileum contained numerous tubercular ulcers and nodules on the mucous membrane; the

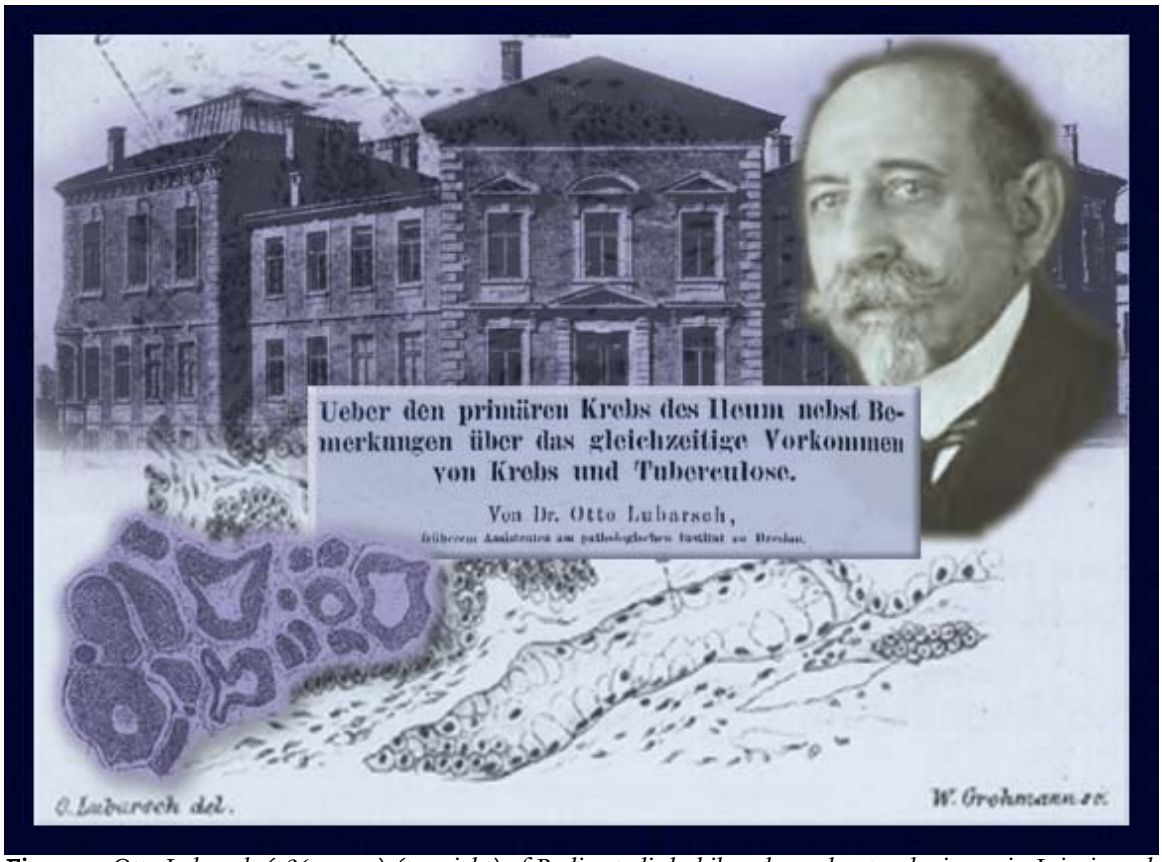


Figure 2*: Otto Lubarsch (1860-1933) (top right) of Berlin studied philosophy and natural sciences in Leipzig and Heidelberg and later earned his medical degree in Strasbourg in 1883. After working as an assistant at the Institute of Physiology in Bern (top background) and at the Pathological Institutes of Giessen, Breslau, and Zurich, he became Professor of Anatomy and Pathology at the Pathological Institute in Rostock in 1894. Well-known for his eponymous characterization of tiny crystals found in the epithelial cells of the testis that resemble sperm crystals ("Lubarsch' crystals") and Lubarsch-Pick syndrome (systemic amyloidosis, primary, systematized amyloidosis, systematized amyloidosis with macroglossia), he also provided the first detailed pathological description (lower left, background) of carcinoid tumors while in Breslau in 1888, in which he reported multiple ileal carcinoid tumors on autopsy in two male patients (frontispiece, center).*

carcinomatous growths in the ileum and a cirrhosed liver. Interestingly, diarrhea had been a prominent symptom in the latter patient, quite possibly as a manifestation of carcinoid syndrome, though Lubarsch made no mention of metastatic disease in either case. Microscopically, he noted a low-grade penetration of the tumor into the muscularis circularis and hyperplasia of the muscularis mucosae. Lubarsch was reluctant to identify these lesions

as carcinomas, and after a careful search through the world's literature he found records of thirty-five cases of intestinal carcinomas occurring near the ileocecal valve. He was of the opinion that several of these were not true carcinomas.

William Bramwell Ransom of Nottingham was the third to describe a lesion resembling carcinoid tumors[19]. In the November 1890 issue of *The Lancet*, Ransom described a fifty-year-old woman who initially presented with two egg-sized lumps in the lower part of her stomach, menorrhagia, and severe diarrhea. Her symptomatology was mostly attributed to uterine fibroids, and her symptoms had only somewhat improved by the time she left the hospital several weeks later. The diarrhea persisted for the next two years, after which she presented with a large, palpable abdominal mass and substantial cachexia. Of particular interest was the observation of severe attacks of wheezing and diarrhea upon eating, arguably the first reported presence of carcinoid syndrome, which had hitherto remained undocumented. Upon her death soon thereafter, autopsy revealed several small nodules in the ileum, six inches above the ileocecal valve, along with extensive hepatic metastases. The nodules were walnut-sized, and the tumor demonstrated rounded, polypoid growth patterns without any ulceration or macroscopic changes to the mucosa. The tumor cells were arranged in small nests that formed small tubes or solid bars, and hardly any remains of villi or crypts of Lieberkühn were visible near the free surfaces of the tumor. As with the two cases described by Lubarsch, Ransom noticed a hyperplasia of the muscularis mucosae. Ransom astutely noted that these tumors resembled carcinomas only histologically, may remain undetected for a long time, and generally demonstrated very slight, local malignancy or a tendency to infiltrate or destroy their surrounding tissues. He proposed that their malignant potential was largely dependent on the local conditions (i.e.

vascular supply, resistance) of the peri-tumoral tissues. He referred to the tumors as "glandular carcinomas", although their peculiar appearance caused by penetrating blood vessels into the tumor led him to propose a resemblance to cylindromas (uncommon benign adnexal tumors). In fact, he believed this angiogenesis was more consistent with the behavior of sarcomas than with carcinomas. Ransom could only speculate, however, on the etiology of these tumors and no definitive conclusions were made.

In 1895, A. Notthafft, an assistant at the Pathological Institute of Würzburg, described three tumors of the upper jejunum found at autopsy in a patient who had died of pneumonia. The first "pinhead-sized" tumor was found approximately 10 cm past the end of the duodenum; the second and third tumors, both "pea-sized", were found 10 cm and 15 cm distal to the first tumor, respectively. They had a whitish color, hard consistency, and a smooth surface. He noted rampant tumor growth in the submucosa, which rose strongly over the level of the adjacent mucous membrane, and there was small cellular infiltration in much of the adjacent mucous membranes and into the muscular layer. The tumors were uncharacteristically identified in the submucosa and histologically not true carcinomas; he thus referred to them as "beginning carcinomas"[20].

The carcinoid tumor then faded into obscurity once more, and twelve more years passed before Oberndorfer's initial unsuccessful attempt to characterize and delineate the properties of this enigmatic neoplasm was presented.

Oberndorfer and Karzinoide

Oberndorfer first presented his observations on carcinoid tumors at the German Pathological Society convention in Dresden in September 1907 and in December of the same

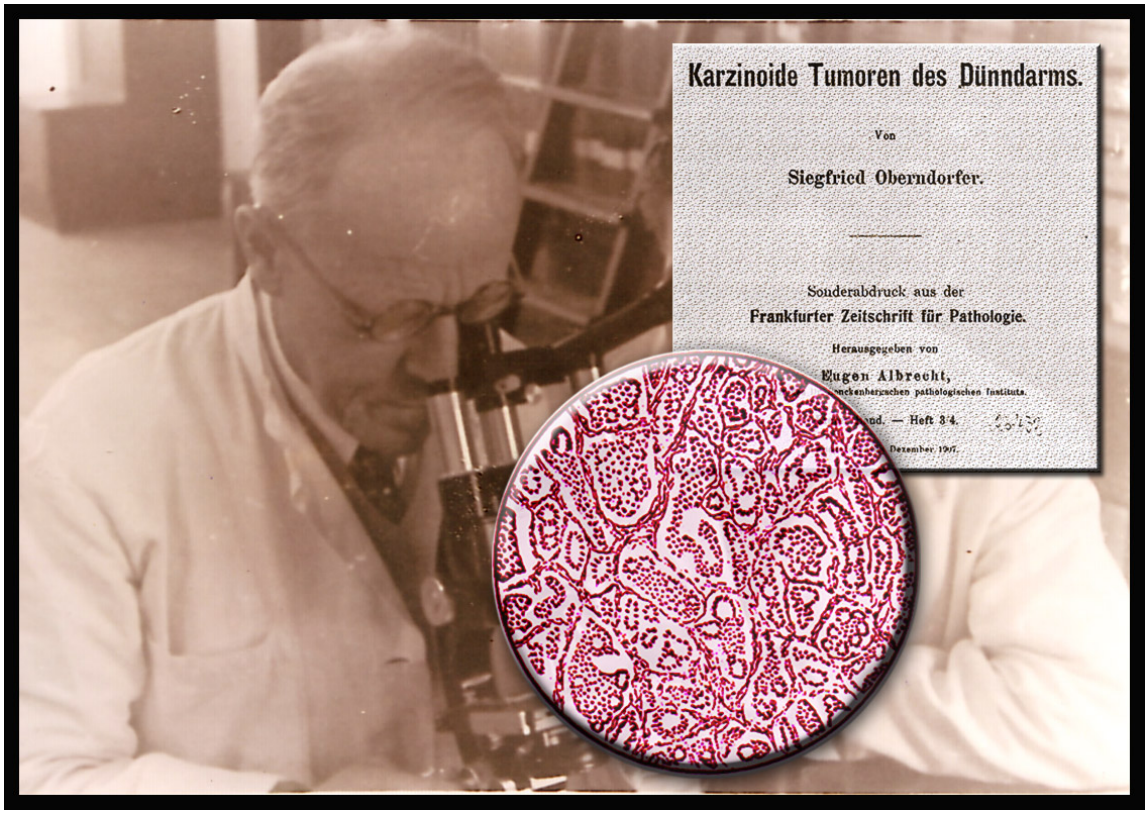


Figure 3: *In September of 1907, Oberndorfer first presented his seminal work on carcinoid tumors at the German Pathological Society meeting in Dresden, which he published three months later in the Frankfurt Journal of Pathology (frontispiece, above right). He described six cases of multiple pea-sized ileal tumors in close proximity, which grew extremely slowly and presumably without any metastatic potential (an observation he later refuted in 1929). Upon microscopic evaluation (lower right), however, the tumors featured the histological characteristics of carcinomas (e.g. undifferentiated tissues surrounding the tumor cells). Oberndorfer successfully demonstrated that this seemingly paradoxical finding was indeed a novel disease entity, and he assigned the name karzinoide ("carcinoma-like") to more accurately describe these tumors.*

year, published his seminal paper "Carcinoid Tumors of the Small Intestine" in the *Frankfurt Journal of Pathology*[21]. This manuscript was the first to describe and characterize the tumor that had previously been referred to as a "benign carcinoma" (*Figure 3*).

The first case described a 48-year-old woman who had been presumed to have perished as a result of tuberculosis. At autopsy, four pea-sized tumors were found in the ileum, three of which were separated by approximately 20 cm along the intestine, while the fourth tumor was only 1 cm distal to the third. Each tumor was found in the submucosa, and

the surrounding intestinal mucosa and neighboring serosa revealed no reactive inflammation. The histological findings were consistent with those described by previous authors, in that the lesions were arranged in nests of small polymorphic cells with large nuclei and scant cytoplasm; there were distinguishable, albeit atrophic, crypts of Lieberkühn; dense, fibrous connective tissue comprising the surrounding stroma and rampant epithelial vascular growth was adjacent to the tumor. The mucosa and muscularis mucosae were completely intact, and no cellular infiltration of the tumor into the surrounding stroma could be observed. In addition, noticeable changes of the liver, kidney, and spleen were observed, all of which exhibited high-grade amyloid degeneration, though it was unknown whether or not such findings could be attributed to metastatic carcinoid disease or tuberculosis.

The second case involved a 30-year-old woman who had soon after the birth of a child had died of typhoid fever. At autopsy, three small tumors, approximately the size of peas, were found in the ileum. The first and second tumors were separated by approximately 30 cm, while the third was about 40-50 cm distal to the second tumor. The surrounding stroma of each tumor was comprised of connective tissue and smooth muscle fibers, and proliferation of glandular epithelium was noted. The four other cases reported similar findings, and Oberndorfer noted some general trends. All tumors were located in the submucosa of the ileum, the normal elements of the mucosa (particularly the crypts of Lieberkühn) were almost completely absent, and the stromal tissue consisted of smooth muscle fibers and connective tissue.

Although five of the six cases demonstrated undifferentiated tumor cells that would generally be regarded as carcinomas, Oberndorfer recognized that their clinical behavior was inconsistent with that of a “classical” carcinoma and concluded that these lesions were a

completely different clinical entity. He argued that large carcinomas of the small intestine were extremely rare and he had never observed multiple large carcinomas of the small intestine. He further asserted that a multiplicity of large identical tumors in the small intestine had not previously been reported, and the considerable distance between the tumors suggested that they were each primaries, since "metastatic hematogenous or lymphatic spread could not account for the observed findings". He further reasoned that if the tumors were to be considered as multiple primary carcinomas of the intestine, they must adhere to the three criteria proposed by Michelsohn[22] in that:

1. The new formations must differ from each other morphologically and histologically.
2. Each carcinoma must be histogenetically derived from the epithelium of the mucosa.
3. Every tumor has the potential for metastasizing.

According to Oberndorfer, only the second criterion was fulfilled, and the third criterion was omitted since metastases were not detectable in the cases he had examined. Furthermore, the tendency of these tumors to grow very slowly distinguished them from the rapid, highly invasive nature of carcinomas. As a result of his observations, Oberndorfer ascribed five distinct characteristics to these tumors:

1. They are mostly small, with patients commonly demonstrating multiple tumors.
2. The tumor cells are usually surrounded by undifferentiated tissues, possibly demonstrating gland formation.
3. The tumors have not previously been described, and they have the potential to become invasive.
4. They do not metastasize.

5. They apparently grow extremely slowly, achieving no substantial size, and therefore appear to have a harmless nature.

Given their "aberrant" characteristics, Oberndorfer asserted that such tumors could not be categorized as any other small intestinal neoplasm and therefore represented a novel disease entity.

Of paramount importance to Oberndorfer was determining whether these tumors were actually true cancers. Although their histological appearance was consistent with a malignant process, the clinical features suggested otherwise, as the lesions did not demonstrate rapid growth, the tumor borders were sharply circumscribed, and they did not appear to metastasize. Given that the classical understanding of a carcinoma did not appear to encompass the behavior of the processes that he had observed in these tumors, Oberndorfer reasoned that perhaps the term *karzinoide* ("carcinoma-like") might more accurately describe the lesions. Although Oberndorfer's early contributions to the understanding of the biology of carcinoid tumors were prescient, his assertion that the tumors were of a benign nature subsequently proved to be incorrect. However, in 1929, twenty-two years after first characterizing the carcinoid tumor, Oberndorfer described his further experience with thirty-six carcinoid tumors of the appendix and small intestine. In this manuscript, he revised his initial characterization of the benign behavior of the tumor and confirmed the possibility that *karzinoides* might exhibit malignant features and metastasize[23].

Cell of Origin: The Enterochromaffin (EC) Cell

Although progress had been made in elucidating the pathological nature of this "odd" tumor of the small intestine, there was a paucity of information available regarding this tumor's cell of origin. W. M. Bayliss (1860-1924) and E. H. Starling (1866-1927) provided the first scientific evidence that the gut was an endocrine organ in 1902[24]. But much earlier in 1868, R. P. Heidenhain (1834-1897) of Breslau, Prussia found enterochromaffin (EC) cells in the gastric mucosa, and in 1870 he first noted the existence of enterochromaffin-like (ECL) cells, although he was not able to define their role[25]. He noted that such cells, distinguishable by their deep-yellow color when stained with bichromate solutions, were simply morphologically different to other intestinal mucosal epithelial cells. In 1897, Nikolai Kultschitzky of Russia noted similar cells in the crypts of Lieberkuhn in the intestinal mucosa[26]. Similar observations were made by A. Nicolas (1891)[27], H. Kull (1924)[28], and M.C. Ciaccio[29], the latter of whom introduced the term "enterochromaffin" in 1906. In 1914, A. Gosset (1872-1944) and P. Masson (1880-1959), using silver impregnation techniques, demonstrated the argentaffin-staining properties of carcinoid tumors[30] (*Figure 4*) and suggested that these neoplasms might arise from EC cells.

Subsequent studies demonstrated that carcinoid tumor cells indeed displayed characteristic light microscopy and histochemical features in their reactions with silver salts (e.g. argentaffinity and argyrophilia). In 1938, Friederich Feyrter (1895-1973), Professor of Pathology at the Medical Academy of Danzig, Poland, proposed that carcinoid tumors were derived from the diffuse endocrine system[31]. He based this proposal on the observation of argentaffin-positive and argyrophilic "clear cells" (*helle zellen*) throughout the gut and pancreas that failed to take up conventional stains[32]. By 1948, A. B. Dawson had developed a

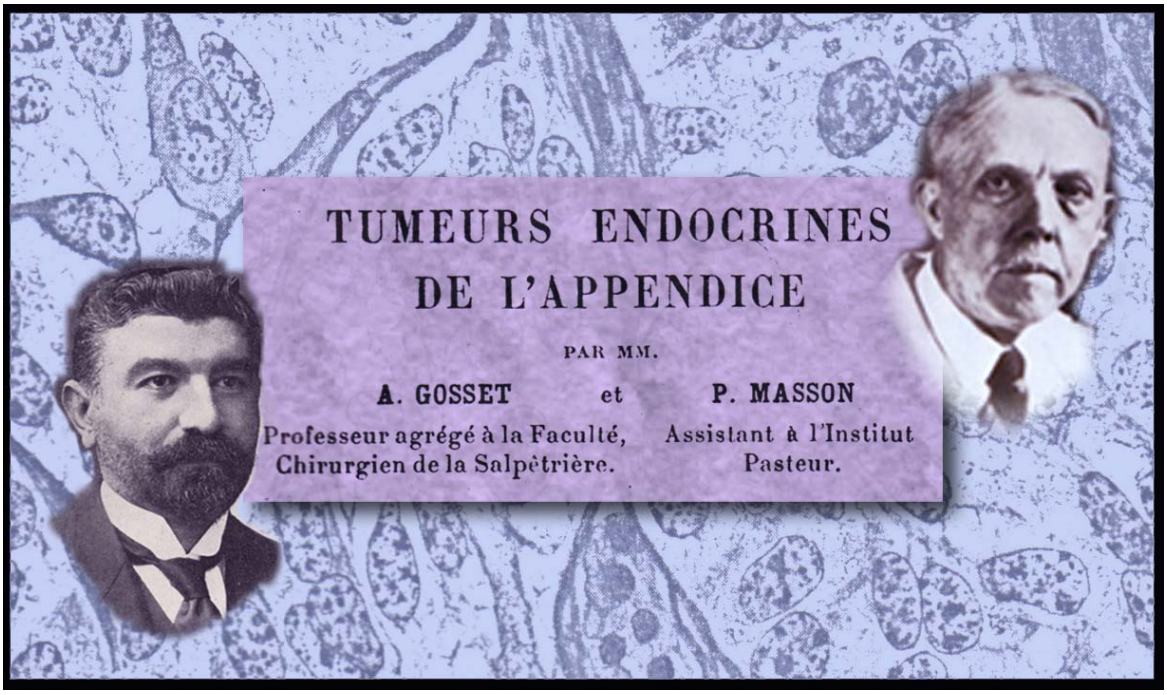


***Figure 4**: Pierre Masson (above right) of Dijon studied medicine in Paris, and after earning his degree in 1909 continued his studies at the Pasteur Institute until 1914. By the end of World War I, his reputation as a histopathologist was so great that, despite his youthful age and inexperience, he was offered the Chair of Pathology at the University of Strasbourg. He attained notoriety with the pioneering development of his eponymous trichome stain, which became the standard in all pathology laboratories. This innovative technique allowed him and A. Gosset (lower left) to demonstrate in 1914 the argentaffin staining properties of carcinoid tumors (frontispiece, center). They suggested that the Kulchitsky, or enterochromaffin (EC) cells in the gut (background), which had been earlier discovered in 1897 by Nikolai Kulchitsky within the crypts of Lieberkühn in the intestinal mucosa, formed a diffuse endocrine organ. In 1928, they described these cells as being of neural origin, and proposed that they were the progenitors of neuroendocrine tumors of the gut (carcinoids). By 1927, Masson was invited by the Université de Montréal to assume the role of Chair of the Department of Pathology, a position he held until his retirement in 1954.*

technique by which EC and ECL cells of the gastrointestinal tract could be stained with silver nitrate[33]. Serotonin, or 5-hydroxytryptamine (5-HT), was described and isolated by M. Rapport in 1948[34]. In 1952, V. Erspamer and B. Asero of Milan, both working in the Department of Pharmacology at the University of Bari, isolated 5-HT in the EC cells of *Octopus* and *Discoglossus* tissues and suggested that "enteramine" (serotonin) was the specific hormone of the EC cell system[35]. The *Octopus vulgaris*, a common native polyp, and

the *Discoglossus pictus*, an amphibian of Sicily and Sardinia, were both readily available and particularly useful because their tissues and organs were especially rich in enteramine. In 1953, F. Lembeck (1922-) of Graz biochemically confirmed the presence of 5-HT in an ileal carcinoid tumor[36], corroborating the assumption that human EC cells contained this bioactive amine.

Histopathological Classification

In 1966, Anthony Pearse (1916-2003) recognized that the endocrine cells of the gut were linked together by a group of common cytochemical characteristics; in particular, the uptake of 5-hydroxytrytophan (5-HTP) and its decarboxylation to 5-HT was analogous in this distinct population of endocrine cells[37]. By 1968, these peptide hormone-producing cells, all of which presumably derived from the neural crest, were collectively known as "amine precursor uptake and Decarboxylation" (APUD) cells[38]. Since that time, the definition of these cells has been modified somewhat, and they are now known as the "diffuse neuroendocrine system" (DNES) (*Figure 5*). Although Pearse and others initially suggested that APUD cells were derived from neural crest cells, it is now generally recognized that gastroenteropancreatic APUD cells probably arise from endoderm[39]. Le Douarin and Dupin[40] later demonstrated the multipotency of neural crest cells and proposed that enteric gangliogenesis by neural crest cells reflected the effects of multiple growth factors of the glial-derived neurotrophic factor family, as well as the endothelin-3/endothelin receptor B pathway. More recently, Chalazonitis *et al.*[41] reported that differentiation of various enteric neurons is also regulated by neurotrophin-3.

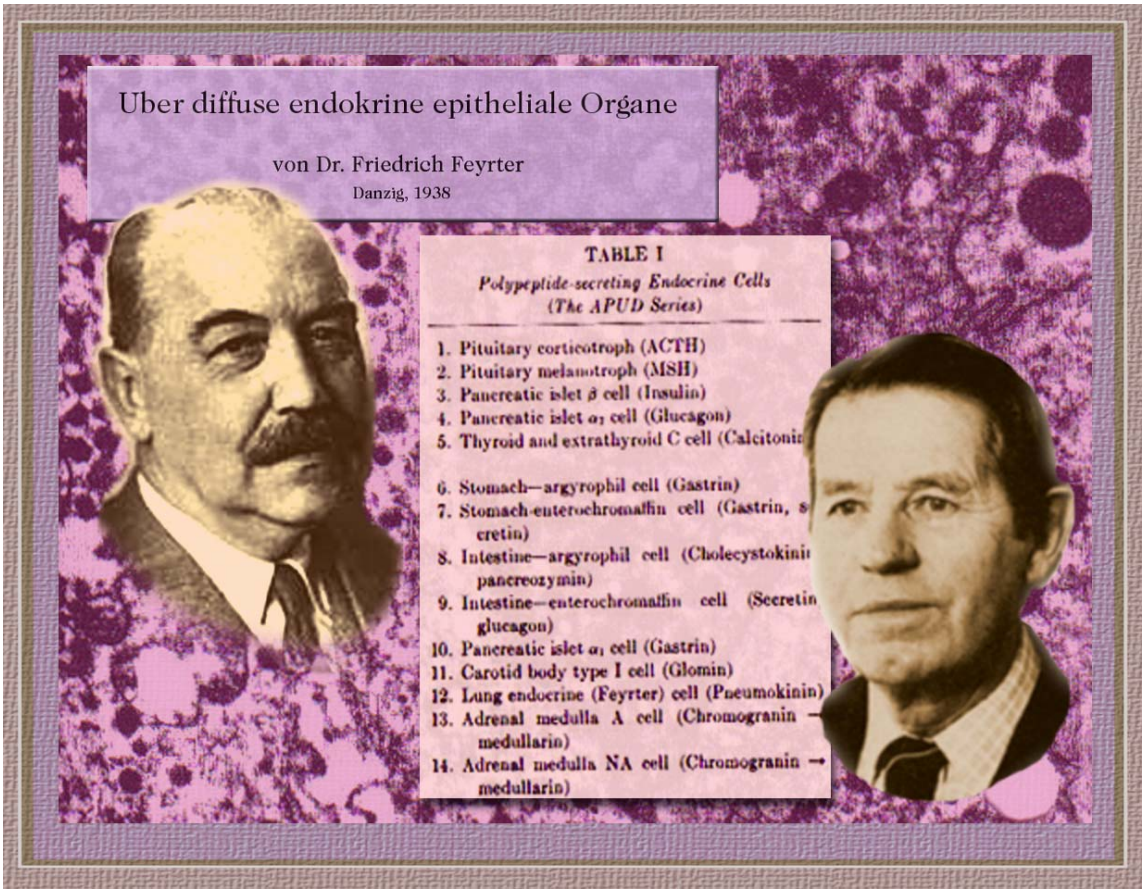


Figure 5*: The first description of what has now come to be known as the "diffuse neuroendocrine system" (DNES) was provided in 1938 by Friedrich Feyrter (left), then Professor of Pathology at the Medical Academy of Danzig. He recognized the presence of argentaffin or argyrophil "clear cells" (helle zellen) in the gut and pancreas, and proposed that such cells were the source of hormones that acted locally (frontispiece, top left). In the 1960s, A.G. Everson Pearse (1916-2003) (below right), a histochemist at the Royal Postgraduate Medical School in London, demonstrated that the cells described by Feyrter shared many functional characteristics with cells in many of the major endocrine glands, such as the thyroid and hypothalamus. All were associated with the metabolism of amines and the secretion of peptides and were thus conveniently described by the acronym APUD (amine precursor uptake and decarboxylation) (center). Pearse's work was instrumental in confirming the concept of a single neuroendocrine system that populated the tissues of the body and was particularly conspicuous in the gastrointestinal tract.*

Another ubiquitous yet inconsistently defined histopathological classification of carcinoid tumors in the literature is the "typical" versus "atypical" designation, usually in reference to their degree of differentiation. "Typical" carcinoids, by definition, are tumors with neuroendocrine differentiation and characteristic histological architecture of trabecular, insular, or ribbon-like cell clusters, with no or minimal cellular pleomorphism and sparse

mitoses[42]. "Atypical" carcinoids, however, demonstrate a more aggressive, poorly-differentiated phenotype with increased mitotic activity and an absence or limited extent of necrosis[43].

Recently, more sophisticated methods of analysis have facilitated the development of precise classification systems that can discern the motley assortment of peptides and amines present in carcinoid tumors. As many as forty different secretory products have been identified in the different varieties of carcinoid tumors[44]. Ultrastructural findings of intracytoplasmic electron-dense secretory granules and immunoreactivity with antibodies to Chromogranin A are useful tools for confirming the diagnosis of carcinoid tumors[45].

Embryological Classification

Carcinoid tumors are commonly classified by their embryonic gut origin; an archaic but somewhat useful distinction, since the features of carcinoid tumors derived from each respective location differ clinically, histologically, and immunochemically. In 1963, E. D. Williams and M. Sandler proposed the original classification of carcinoid tumors based on their putative embryologic origin (e.g. foregut, midgut, or hindgut)[46]. In 1971, J. Soga and Y. Yakuwa introduced a histological classification based purely on morphological characteristics, describing carcinoid tumors according to their dominant growth patterns: insular, trabecular, glandular, mixed, or undifferentiated[47].

Although carcinoid tumors have historically been classified according to the foregut, midgut, or hindgut derivation, this stratification was developed before the elucidation of the different NE cell types and the appreciation that an embryologic classification had little mechanistic or physiological validity[48]. Unfortunately, this historical classification predates

the understanding of the different cell types responsible for the broad group of tumors generically grouped as carcinoids and has thus hindered the appreciation of the divergent biological and pathological behavior of the individual tumors and their various respective secreted peptides and amines. Broadly speaking, it has been accepted that foregut endocrine cells give rise to carcinoid tumors in the respiratory tract, stomach, first part of the duodenum, and pancreas; midgut carcinoid tumors represent lesions of the bowel from the second part of the duodenum through the ascending colon and appendix; and hindgut carcinoids constitute lesions of the transverse and descending colon and rectum. Carcinoid tumors from different segments of the embryologic gut typically vary widely in terms of the character of their bioactive products, and these differences result in a wide range of clinical symptoms and immunohistochemical staining patterns. Variations in anatomic location and venous drainage may further alter the clinical presentation. Midgut tumors most commonly produce serotonin and tachykinins and often cause systemic symptoms (e.g. diarrhea, flushing, wheezing, right-sided valvular disease, and cutaneous telangiectasia) once the tumor has metastasized to the liver, or in rare instances following drainage of the tumor's bioactive peptides directly into the systemic circulation. An exception to this is the appendiceal carcinoid, which is a relatively benign lesion (more neural than endocrine in its biological behavior) that rarely produces serotonin[49].

Epidemiology

Carcinoid tumors are relatively uncommon, with an overall incidence of 1-to-2 per 100,000 people in the United States and comprise only 0.49% of all malignancies[51]. A recent series of nearly 14,000 carcinoids noted that the overall male-to-female ratio for carcinoid of

the small intestine is 1.1:1[51]. Incidence among white males is 0.88 per 100,000 people per year (1.65 among African-American males), while incidence among white females is somewhat lower, with an analogous increased incidence among African-American females: 0.63 and 1.15 per 100,000 people per year, respectively[51].

In contrast to the better survival rates for carcinoids of the stomach, duodenum or rectum (>80%), patients with jejuno-ileal carcinoids have a relatively poor 5-year survival rate (55%)[51]. The discrepancy is probably related to the earlier detection of the former, as the symptomatology of any local lesion (dyspepsia or bleeding) leads to endoscopy, while EC carcinoids do not usually become symptomatic until they have metastasized and actively secrete bioactive products into the systemic circulation[50]. The poor prognosis also reflects the malignant behavior of the tumor with a high propensity to disseminate to both lymph nodes and the liver.

Over 60% of carcinoids arise along the gastrointestinal tract; particularly the small intestine, appendix, and colon[51], a reflection of the dense neuroendocrine cell population of the gut and the conglomeration of "regulatory cells" necessary to sample and regulate the gut environmental milieu (*Figure 6*). Similarly, the bronchopulmonary system possesses a respiratory epithelium densely populated with Kultschitsky cells and accounts for approximately 25% of carcinoid tumors, comprising roughly 2% of all primary lung tumors[52].

Because of their slow-growing nature, most carcinoid tumors never spread beyond the confines of the primary lesion and are often found incidentally during surgery or autopsy[4]. However, despite their indolent behavior, these lesions are by no means benign and are histologically similar to carcinomas, which have the potential to become invasive and

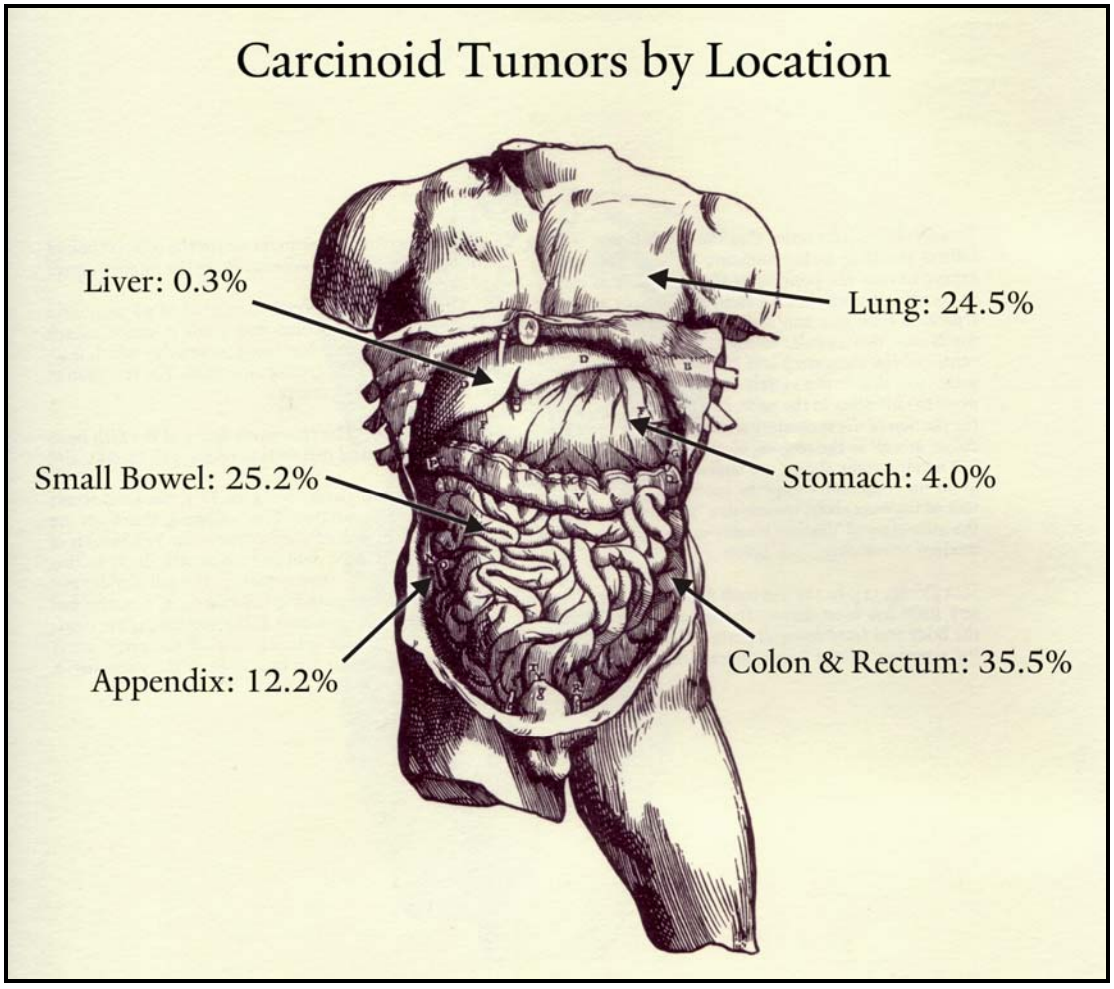


Figure 6: *The majority of carcinoid tumors (over 60%) are found in the gastrointestinal tract, with the lung being the second most common site (24.5%) of primary tumors.*

give rise to extensive nodal and hepatic metastases. Their metastatic potential has been found to be directly proportional to the size of the primary lesion, with only 2% of small bowel lesions less than 1 cm in diameter showing metastases in one series, while approximately 80% of tumors with a diameter of 2 cm or more demonstrated metastases[53]. A recent series noted that 44% of patients with primary tumors less than 1 cm in diameter had evidence of metastatic spread to the lymph nodes, while those with tumors 1-2 cm and >2 cm in size demonstrated evidence of lymphatic metastatic spread in 77% and 85% of cases,

respectively[54]. The most common initial diagnosis among patients with small bowel carcinoids is mechanical small bowel obstruction of unspecified cause, which occurs in 20-25% of patients with these tumors[55,56].

Carcinoids and Clinicians

Carcinoid Syndrome

The constellation of symptoms including, but not limited to, edema, flushing, and diarrhea, now commonly referred to as the "carcinoid syndrome", was first described in 1931 by A. J. Scholte[57], a Dutch pathologist who documented at autopsy a 1 cm ileal carcinoid tumor in a 47-year-old male who had suffered from diarrhea, cyanosis, cough, lower extremity edema, and cutaneous telangiectasia before dying from cardiac failure and bronchopneumonia. In the same year, M. A. Cassidy reported a patient who complained of flushing and diarrhea before his death; autopsy revealed the presence of metastatic "adenocarcinoma" and cardiac valvular lesions[58]. Although Cassidy did not provide an adequate histological description of the tumor tissue to confirm that these lesions were carcinoids, his manuscript included a picture of the patient's face that clearly illustrates a flushed appearance consistent with the carcinoid syndrome (*Figure 7*). In 1943, S. Millman described a 44-year-old female patient with flushing who on autopsy was found to have multiple ileal carcinoids with metastatic spread to the liver and lymph nodes[59]. In 1954, A. Thorson and his colleagues[60] of Malmö, Sweden published the first series of patients presenting with pulmonary stenosis, tricuspid insufficiency, peripheral vasomotor symptoms, bronchoconstriction, and cyanosis in association with malignant carcinoid tumors of the small intestine with metastatic spread to the liver. The report presented 7 "definite" cases, 4 "probable" cases, and 5 cases with partial or not fully verified symptoms, and their symptomatology was related to the hypersecretion of 5-HT from the carcinoid tumors into the systemic circulation. In the same year, B. Pernow and J. Waldenström, also of Sweden,

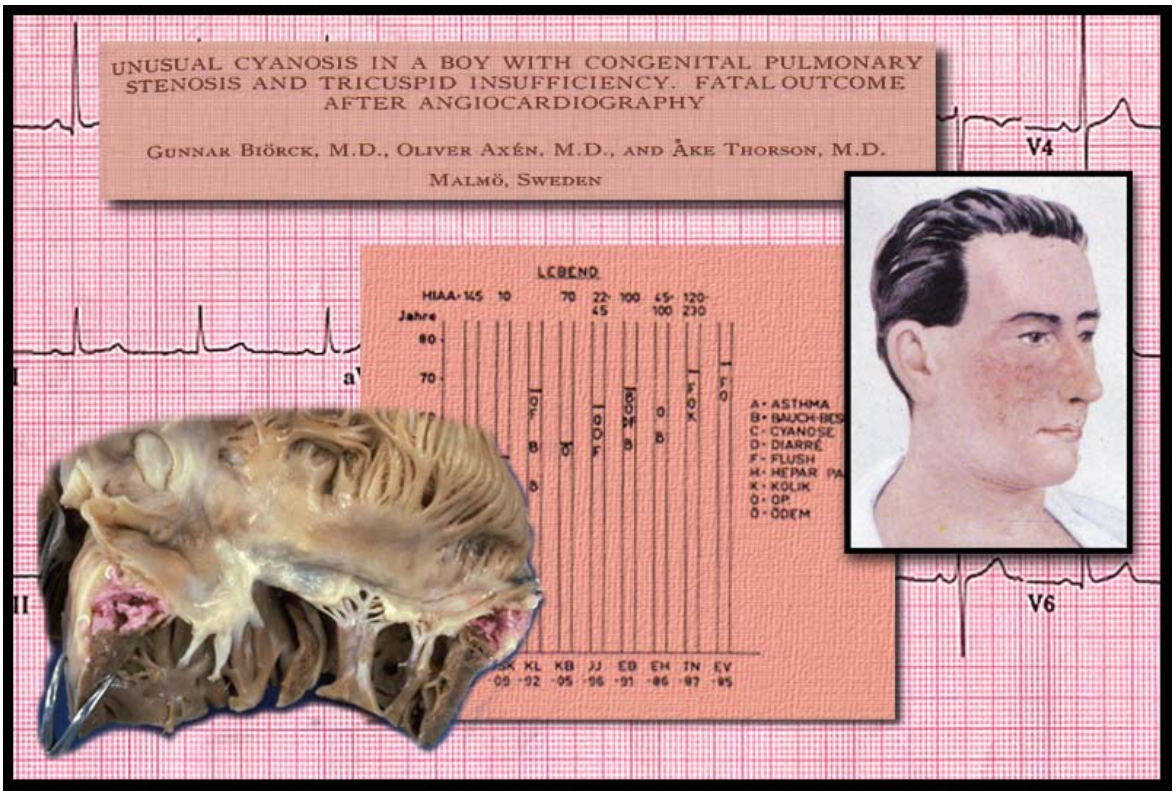


Figure 7*: Cassidy in 1931 described a patient who complained of flushing and diarrhea; a picture of his patient (right) demonstrates the classic facial flushing associated with carcinoid syndrome. Biörck and his colleagues of Sweden were among the first to describe the carcinoid syndrome ("carcinoidosis") in 1952 (frontispiece, top), which they characterized as a constellation of symptoms associated with abnormally high levels of plasma serotonin (center) including flushing, diarrhea, edema, wheezing, and right-sided heart failure, the latter of which results from the deposition of fibrotic subendocardial plaques (bottom left; fibrotic plaques stained pink) and is commonly referred to as "carcinoid heart disease".*

added paroxysmal flushing as a key component of this syndrome[61]. In 1964, J. A. Oates demonstrated that some carcinoid tumors release kallikrein (which activates bradykinin, a potent vasodilator) and suggested that kallikrein might also play a role in the flushing episodes so characteristic of the disease[62].

In 1952, G. Biörck described carcinoid heart disease in a 19-year-old boy suffering from pulmonary stenosis with tricuspid insufficiency and cyanosis[63]. The patient was dyspneic at rest and died while undergoing an angiogram, with electrocardiography

demonstrating slow contractions of a very broad bundle branch block type. Autopsy revealed a malignant carcinoid in the jejunum with extensive hepatic metastases. This report was among the first to describe both pulmonary stenosis and tricuspid insufficiency in the presence of carcinoid tumors.

Carcinoid syndrome is reported to occur in up to 18% of patients with midgut carcinoids[64]. The presence of the carcinoid syndrome generally implies that the patient has liver metastases[65-67], although symptoms may also occur at earlier disease stages owing to mesenteric metastases and fibrosis, which may cause obstruction and ischemia of the intestine[65]. Extensive liver metastases without the presence of carcinoid syndrome, though rare, may occur, suggesting a non-secretory behavior of certain tumors that may not necessarily be of EC cell origin. An association with other non-carcinoid neoplasms is evident in 16.6% of distal small bowel carcinoids and constitutes the largest percentage of such co-morbidity among all gastrointestinal carcinoids[68]. This aspect of the lesion presumably reflects its production of growth factors that regulate cell proliferation.

Biochemical Markers

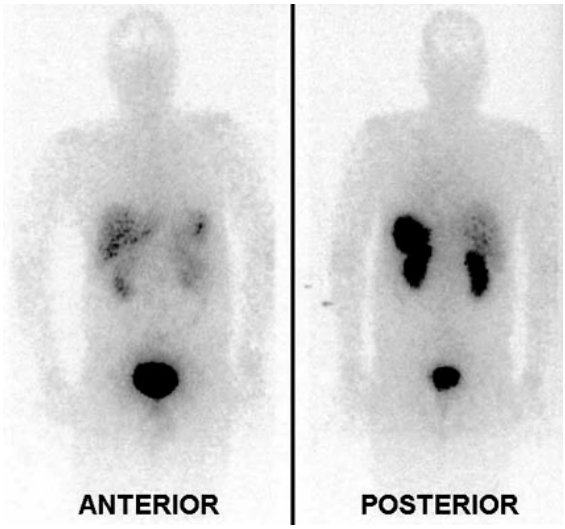
Carcinoid tumor cells are densely packed with neurosecretory granules containing various hormones and biogenic amines. Carcinoid tumors can be diagnosed and monitored by measuring a variety of bioactive peptides that are commonly secreted from the tumors into the blood, including serotonin (5-HT), Chromogranin A (CgA), substance P, histamine, dopamine, neurotensin, prostaglandins and kallikrein. The measurement of general and specific biochemical markers in patients with carcinoids gives an indication of the effectiveness of treatment and may be used as prognostic indicators[69].

The utility of 5-HT as a plasma marker for carcinoid syndrome was first demonstrated by I. H. Page in 1954, based on his observation of elevated urinary excretion of the main 5-HT metabolite, 5-HIAA, in a patient with carcinoid syndrome[70]. Subsequently in 1956, B. J. Haverback and A. Sjoerdsma confirmed this finding in a series of 11 patients with carcinoid syndrome, all of whom displayed markedly elevated levels of urinary 5-HIAA over a 24-hour period[71]. In 1957, B. Pernow of Stockholm provided definitive evidence to explain this phenomenon by measuring serum 5-HT and urinary 5-HIAA levels in 33 patients with abdominal carcinoid tumors[72]. Among the patients examined before operation or after complete tumor resection, increased serum or urinary 5-HT levels were noted in 17 of 18 cases, and abnormally high 5-HIAA levels were found in 19 of 20 cases.

CgA is a member of the chromogranin family, which consists of at least three different water-soluble acidic glycoproteins (CgA, CgB and CgC) stored in the secretory granules of NE cells. CgA is a constitutive secretory product of most NETs. Its detection in plasma can be utilized as a general tumor marker for carcinoids, and its concentration correlates with tumor burden[73]. Plasma CgA levels are sensitive but non-specific markers of carcinoid tumors since they are also elevated in pancreatic NE tumors, as well as in other types of NE tumors[74]. Elevated CgA concentrations are not always specific for NETs, as prostatic carcinoma can be associated with elevated CgA concentrations. However, current assessment of prostatic tumors suggests that some lesions may have a substantial NE component[75]. False-positive increased CgA concentrations can be seen in renal impairment, liver failure, atrophic gastritis and inflammatory bowel disease[76]. Exercise, trauma-induced physical stress, or untreated hypertension can also produce higher concentrations of CgA than in the normal, resting state[73].

Diagnostic Imaging

Carcinoid tumors have high concentrations of somatostatin receptors and can therefore be imaged and specifically localized with somatostatin-receptor scintigraphy using a radiolabeled form of the somatostatin analogue octreotide (^{111}indium pentetreotide)[77]. This diagnostic modality, commonly known as the Octreoscan®, has the advantage of instantaneous whole body scanning, which also allows detection of distant metastases. The overall sensitivity of Octreoscan® is about 80% to 90%[78], and it is effective in detecting primary and metastatic lesions not apparent by conventional radiological imaging techniques (*Figures 8 and 9*).



Figures 8 and 9: *Octreoscan® in a patient with metastatic carcinoid disease. Radiolabeled octreotide binds to somatostatin receptors, which are found in abundance on carcinoid tumor cells, and enables clinicians to detect the presence and extent of carcinoid disease. The extent of widespread disease, as seen above in this patient with multiple hepatic metastases, can be visualized fully and quickly with this diagnostic modality.*

Figure 10: *Gastric carcinoid tumor, as visualized by upper endoscopy. Photo obtained with permission from Dr. Irvin Modlin.*

Alternatively, upper GI endoscopy can identify lesions as far as

the ligament of Treitz and lower endoscopy can detect some terminal ileal tumors as well as colorectal carcinoids (*Figure 10*). Luminal examination has been augmented by computer-assisted tomography (CT) scanning and magnetic resonance imaging (MRI). In the last decade, however, the Octreoscan® has become the most widely used diagnostic technique and should be used as the primary imaging method in patients with carcinoid tumors.

The presence of carcinoid heart disease can be assessed with transthoracic echocardiography. Thickening and retraction of immobile tricuspid valve leaflets with associated severe tricuspid valve regurgitation are characteristic echocardiographic features of advanced carcinoid heart disease[79].

Treatment Strategies

Patients with small bowel carcinoid tumors generally have poorer 5-year survival rates than those with carcinoid tumors in other GI locations[160]. This reflects both the relatively aggressive nature of small intestinal EC cell proliferation and the cryptic nature of their initial clinical presentation. Surgical, interventional radiological, pharmacological, and chemotherapeutic strategies have all been employed in the treatment of patients with carcinoids, but for patients with fibrosis, surgery has thus far been the most logical choice since no agents exist that are able to alter the progression of fibrosis.

Recently, the management of carcinoid tumors with pharmacological and chemotherapeutic agents has increased patient survival. The somatostatin analogue octreotide, a synthetic octapeptide that binds to the somatostatin subtype-2 receptor, inhibits the secretion of bioactive substances, most notably serotonin, that cause the carcinoid syndrome[79]. The presence of functional somatostatin receptors in carcinoid tumors, and the

suppressive effect of somatostatin on the active substances secreted by these tumors (e.g. serotonin, tachykinins), provides the pharmacological rationale for using somatostatin analogues to treat the symptoms associated with carcinoid syndrome[80]. While treatment with octreotide relieves symptoms in more than 70% of patients[81], this therapy does not prevent the progression of cardiac fibrotic lesions, which developed in six of nine patients treated in a recent study[90]. Furthermore, many patients become refractory to this palliative therapy over time[82], and despite a dramatic amelioration in symptoms there are no reports that somatostatin analogues have any influence on fibrosis. In addition, a combination of octreotide and hepatic artery embolization had no significant effect on the development or progression of these lesions[90]. Of note was the fact that this study also identified that patients treated with cytotoxic chemotherapy exhibit an increased risk of progressive carcinoid-related cardiac lesions[90].

For inoperable, well-differentiated, metastatic neuroendocrine tumors, biotherapy with somatostatin analogs or interferon-alpha is the treatment of choice[83], which is able to control both symptoms in functional tumor disease and tumor proliferation. Chemotherapy has been limited to well-differentiated tumors of the foregut, which are relatively sensitive to this treatment mode, and to the rarer, undifferentiated neuroendocrine tumors which are often unresponsive to biotherapy[84,85]. In a recent prospective, randomized, multicenter study, patients with metastatic gastroenteropancreatic tumors demonstrated similar responses (partial remission or stable disease over a 12 month period) when treated with either somatostatin (32%), interferon-alpha (30%) and a combination of the two (25%)[86]. Interestingly, there were no differences in outcome for either functional or non-functional disease. Progression of disease was, however, noted in greater than 50% of patients.

The only current therapy for fibrotic carcinoid tumors or carcinoid heart disease is surgery[92]. In one study, a dissectional removal of mesenteric tumors resulted in a substantial symptomatic relief [92]. More often than not this is impossible and leads to multiple enterotomies or mesenteric devascularization and ischemic bowel infarction. Surgical debulking of the hepatic disease has been shown to improve survival[87], but current recommendations indicate that the removal of 90% of the disease is required to achieve palliation in these patients[88]. In one recent retrospective examination of patients undergoing surgery at the Mayo Clinic[89], it was found that although an operation controlled symptoms in 104 out of 108 patients, the recurrence rate was 59% at 5 years, and the five-year survival was 35%. Surgical curative treatment of neuroendocrine tumor disease can only be achieved in patients with small primary tumors or tumors with limited local disease.

While cardiac valvular surgery may be appropriate for patients with carcinoid heart disease in certain cases, a hepatic artery embolization approach failed to significantly affect carcinoid heart disease in one study[90]. The direct dissectional approach and targeting the liver metastasis will both conceivably decrease the biochemical symptomatology associated with the disease, but the long-term effects on fibrosis are not known.

Carcinoid Tumors and Fibrosis

Overview of Carcinoid-Related Fibrosis

Because of their inconspicuous size and deep submucosal location, primary carcinoid tumors are rarely diagnosed before metastases have developed and patients thus often present with advanced disease. When these tumors manifest clinically, it is most commonly the result of extensive fibrosis around the tumor that often extends throughout the peritoneal cavity. Such fibrosis ultimately leads to intestinal obstruction caused by either kinking of fibrous adhesions of intestinal loops, luminal obstruction by fibrosis within the tumor, or intussusception—all of which warrant surgical intervention[91] (*Figure 11*).

Fibrosis within the abdomen may also contribute to significant bowel ischemia and infarction. Patients with carcinoid tumors often suffer from intestinal venous ischemia and partial or complete intestinal obstruction, commonly associated with abdominal pain, aggravated diarrhea, malnutrition, and general malaise[92]. The fibrosis around mesenteric metastases often causes shrinkage and fixation of the ileal mesentery to the retroperitoneum, with fibrous bands of tumor often attaching to and possibly obstructing the small intestine and transverse colon[92]. Furthermore, the consequences of retroperitoneal fibrosis may include stenosis of the ureters, with associated hydronephrosis and renal failure in several cases[93,94]. Marked vascular damage from the fibrosis may occur if mesenteric vessels and nerves become trapped in dense deposits of peri-tumoral fibrous tissue, and this may lead to bowel (particularly small bowel) ischemia[91] (*Figures 12 and 13*).

The relationship between small bowel carcinoids and fibrosis has been well documented in the literature[92,95-101], yet the mechanism by which the tumors stimulate fibrosis

remains poorly understood. Moertel and his colleagues first described the unique relationship between carcinoids and fibrosis in 1961[95], with particular attention to the fact that obstruction is usually the initial symptom in most patients with carcinoid tumors of the small intestine. Ohrvall further noted that approximately half of the patients with metastatic carcinoids initially presented with and required surgery for intestinal obstruction or acute abdominal pain, often with an unknown diagnosis[92]. In contrast, Delbridge in 1996 asserted that many surgeons faced with the patient with metastatic midgut carcinoid disease should take a nihilistic approach and only reluctantly offer surgery as a form of therapy, opting instead to wait until the development of severe symptoms of obstruction or ischemia before contemplating laparotomy[102]. Thus, while carcinoid tumors themselves are usually (but not always) slow-growing, the fibrosis associated with these tumors eventually leads to major complications that require surgical intervention and accounts for the significant morbidity and mortality associated with carcinoid disease.

Incidence of Carcinoid-Related Fibrosis

Moertel and his colleagues presented one of the earliest retrospective analyses midgut carcinoid tumors with his series of 209 cases[95]. Of these, one hundred and thirty-seven carcinoid cases (65.5%) were identified at autopsy and 72 cases (34.5%) were identified at laparotomy. In Morgan's series of 37 patients with jejuno-ileal carcinoids, 8 of 12 patients with obstruction had fibrosis or bowel kinking[103]. The chief, and sometimes only, clinical symptom that patients presented with were the result of partial or intermittent intestinal obstruction, and 48 of 56 patients (85.7%) who presented with symptomatic lesions had metastatic disease at diagnosis. In a series of 262 patients with carcinoid tumors who

underwent surgery before diagnosis, 121 (46%) presented with severe abdominal pain and intestinal obstruction[104]. In a review of 36 patients with small bowel carcinoids at Yale University (1972-2001), fifteen patients (42%) either subsequently developed or had documented fibrosis at time of operation[105].

Clinical Features

Symptoms of bowel obstruction are not always due to peritoneal fibrosis or an obstructing tumor, and this caveat should be borne in mind when interpreting medical versus surgical series. In the latter, the etiopathogenesis of the pain can often only be directly proven by exploration. In a surgical series of 121 patients with midgut carcinoid tumors, 75 (61.9%) developed abdominal pain and required laparotomy; among these patients, marked mesenteric fibrosis was detected at surgery in 59 (78.6%)[104]. The mesenteric fibrosis among these patients was generally accompanied by symptoms of intestinal obstruction, including feeding-related or crampy abdominal pain, cessation of diarrhea, and weight loss. Of these patients, 90% suffered from acute abdominal pain, 58% developed weight loss (>9 kg), 56% had a palpable lower abdominal mass, and 39% had experienced cessation of diarrhea even leading to episodes of constipation.

In medical studies, abdominal pain was the initial symptom in most patients, described as episodic, colicky pain associated with distension and characteristic of intermittent intestinal obstruction[157]. Cai and his colleagues described a case of ileal carcinoid in a 74-year-old woman who presented with a small bowel obstruction requiring resection[106]; she reported abdominal pain of one month's duration and her clinical exam revealed a palpable mass in her right lower quadrant.

Approximately 5% of midgut carcinoid patients exhibit military seeding in the intra abdominal cavity, reflecting the facility with which carcinoid tumors can seed and grow locally. Many of these patients develop a "frozen" abdomen and pelvis, despite the absence of bulky liver metastases[104]. Although this disease form frequently presents with obstruction, it is not necessarily always associated with fibrosis.

Retroperitoneal Fibrosis

Once the primary tumor invades the muscular layer of the small bowel and spreads into the peritoneum and mesentery, a considerable fibrotic reaction occurs that may mat or buckle multiple loops of bowel together, often leading to kinking of the bowel, intestinal ischemia, volvulus, or obstruction of the lumen. By this time the patient usually has advanced disease and becomes symptomatic, most often presenting with intermittent, feeding-related abdominal pain, weight loss, and a palpable abdominal mass[53,106]. At this point, surgical attention is imperative, since the fibrosis associated with the tumors tends to cause compression and sclerosis of the mesenteric vessels, with one-third of patients with advanced midgut carcinoids subjected to laparotomy demonstrating intestinal venous ischemia or congestion[92]. Anthony and Drury demonstrated elastic vascular sclerosis in 17 of 25 patients with ileal carcinoids[107]. Surgery has been reported to provide durable, long-term symptom relief and substantial periods of survival among these patients[92]. Among patients with metastatic spread, resection of mesenteric lymph nodes and/or liver metastases resulted in alleviation of symptoms and increased survival in two large series.

Carcinoid Heart Disease

Carcinoid heart disease is a unique and dangerous component of EC tumors. It

represents a serious clinical condition, and one-third of all deaths in patients with carcinoid syndrome are related to right ventricular failure secondary to cardiac morphological changes (e.g. stenosis of the tricuspid and pulmonary valve)[108]. The lesions are located on the mural and valvular endocardium, predominantly in the right side of the heart, and consist of fibroblasts or myofibroblasts and a matrix-rich fibrous stroma devoid of elastic fibers covered by endothelium[109] (*Figure 14*). Right-sided valve dysfunction is attributed to the presence of carcinoid plaques, which cause both thickening and retraction of the valve[110].

The most widely accepted therapy for carcinoid heart disease is surgery[92], which may improve symptoms and longevity, but the scarce data report an early mortality of 35% to 53%[111,112]. However, a recent study reported that the excision of mesenteric tumors resulted in a substantial symptomatic relief[113]. However, more often than not this is not feasible and leads to either multiple enterotomies or mesenteric devascularization and subsequent ischemic bowel infarction. Surgical debulking of hepatic disease has been shown to improve survival[114], but current recommendations indicate that the removal of 90% of the disease is required to achieve palliation[115]. A recent retrospective examination of patients undergoing surgery at the Mayo Clinic[116] noted that although resection "controlled" symptoms in 104 out of 108 patients, the recurrence rate was 59% at 5 years, and the 5-year survival rate was 35%. The authors concluded that surgical curative treatment of neuroendocrine tumor disease can only be achieved in patients with small primary neuroendocrine tumors or tumors with limited local disease. The effect of removal of the primary lesion on the evolution of cardiac heart disease is at this time unclear. The use of balloon pulmonary valvuloplasty in conjunction with cardiac catheterization has been proposed as a palliative measure for symptomatic patients with carcinoid heart disease[117], while the use of hepatic artery

embolization to impede the progression of carcinoid heart disease failed to prevent the development of cardiac lesions in one study[118].

The prevalence of carcinoid heart disease is increasing due to prolonged life expectancy of patients, secondary to improved treatment protocols of carcinoid tumors. The prevalence of cardiac heart disease has been noted to be 20%[119], while abnormal tricuspid function was identified in 33% of patients[3] and valvular lesions seen in 53 of 138 (38%) of cases[120].

Historically, the etiology of the cardiac lesions has been considered to be due to excess serotonin that was not degraded by monoamine oxidase (MAO) in the lungs[53]. However, no clinical studies have rigorously examined the direct relationship between circulating serotonin levels to assess whether the agent is actually responsible for the fibrosis. An initial study in 1985 reported that there was no correlation between 5-hydroxyindoleacetic acid (5-HIAA), the byproduct of serotonin degradation, and the extent of the heart disease[121]. Thereafter, numerous authors have proposed a link between 5-HIAA and/or tachykinins and cardiac fibrosis[122,123]. A cardiac threshold for circulating serotonin of 400-500 μM has been proposed as a minimum concentration required for the development of valvular lesions[125,124]. However, treatment resulting in significant reductions of urinary levels of 5-HIAA, with no regression of the cardiac manifestations in carcinoid syndrome, has been observed[125]. A recent study linking 5-HIAA to carcinoid heart disease speculated that another as yet unidentified agent produced by carcinoid tumors probably participated directly or indirectly in the development of these fibrotic lesions[90]. Thus, although the etiology of both intestinal and cardiac fibrotic lesions remains unknown, it seems plausible that their genesis reflects the biological effects of a common agonist. Any hypothesis relating tumor product to fibrosis in either of these areas

needs to include and reflect, apart from serotonin and tachykinins, the consideration of other growth regulatory products from the tumor cell.

Pulmonary Fibrosis

Pulmonary carcinoids comprise approximately 2% of primary lung tumors[126], and of all carcinoid tumors, 25% are found in the lungs[160]. Pulmonary fibrosis has been reported in association with carcinoid tumors, commonly in the setting of advanced metastatic disease[127,128]. Furthermore, Moss and his colleagues noted that in a series of 50 patients with carcinoid syndrome, 9 (18%) had "idiopathic" pleural thickening although no underlying cause for these pleural abnormalities could be identified[128]. Individuals with bronchial carcinoid tumors can develop left-sided valvular lesions, as the tumors may secrete bioactive agents into pulmonary venous effluent, bypassing the liver and lungs, where amines and peptides are usually metabolized[129].

Carcinoid-Related Scleroderma

Cutaneous flushing, most commonly of the face, neck, and upper chest, are hallmark features of the carcinoid syndrome. This flushing may persist for 10 to 30 minutes and tends to first resolve centrally, producing gyrate and serpiginous patterns[130]. Of interest, however, is cutaneous fibrotic disease, particularly a scleroderma-like manifestation, which was first noted in association with carcinoid syndrome in 1958 in a 42-year-old female with atypical scleroderma and symptoms of carcinoid syndrome[131]. On autopsy she was noted to have an ileal carcinoid tumor. Subsequently a number of reports have confirmed the initial observation and documented the relationship of carcinoid-related scleroderma, mostly

affecting the lower extremities and usually associated with primary tumors of midgut origin[132-136].

The underlying biology of the relationship between small bowel carcinoid tumors and fibrosis (at any site) thus remains unknown but presumably reflects the bioactivity of a secreted systemic mediator.

Radiographic Findings Associated with Carcinoid Fibrosis

The distinctive radiographic findings associated with carcinoid tumors and fibrosis have been described in several series. In a retrospective analysis of computerized tomography (CT) findings in 29 cases of proven mesenteric carcinoid tumors, findings were analyzed with matching histological sections to correlate independently for histological pattern, degree of fibrosis, and calcification within the mass[137]. All calcification was localized within areas of sparsely cellular mature fibrous tissue and the degree of radiating strands detected by CT tended to increase with the degree of fibrosis seen histopathologically[138]. Mesenteric lymph node metastases were evident at surgery or on CT scans in 286 patients (91%) in one series[104]. On abdominal films, mesenteric fibrosis may lead to traction or fixation of the bowel[139]. Angiographic changes are more distinctive, with narrowing or occlusion of the distal ileal arcade and stenosis of the intra-mesenteric arteries characteristic findings[139,140].

Management of Carcinoid Fibrosis

Treatment of patients with midgut carcinoids and their associated fibrosis has been limited mostly to surgery, providing therapeutic and palliative relief to patients and resulting in favorable long-term survival rates. The 5-year survival rate for all patients with operable tumors was 68% in Moertel's series, while only 38% of patients with inoperable metastases

survived five years or more after diagnosis[95]. Among the 59 patients in Makridis' series found to have mesenteric fibrosis at laparotomy, 78% remained free of preoperative symptoms during a mean 4.2 years of follow-up[98]. In Hellman's series, 68.7% of patients demonstrated a reduction in symptoms (e.g. diarrhea, abdominal pain) and improved survival after surgical intervention[104]. In a series of 31 patients with metastatic carcinoid disease, resection of liver metastases prolonged survival and reliably eliminated the incapacitating symptoms of the tumors (i.e. carcinoid syndrome)[141]. More recent data have noted that while the presence of liver metastases at presentation did not appear to influence survival, patients with symptomatic tumors exhibited a 5-year disease-free survival of 46% compared to 72% in asymptomatic patients[142]. This however may simply be a reflection of tumor burden and indicative that the metastatic load *per se* correlates with prognosis.

Intestinal stricture (stenosis) consequent upon superior mesenteric vein (SMV) thrombosis is a relatively infrequent, though important, cause of intestinal obstruction. In general, there is some evidence that immediate anti-coagulant therapy is useful in treating patients with SMV thrombosis (of various etiologies)[143]. However, most carcinoid studies to date have utilized a surgical approach in attempting to treat this phenomenon[92,104]. No prospective studies examining other interventional modalities such as vein grafting or bowel auto-transplantation are currently available.

Biological Basis of Fibrosis

Fibrosis is usually a normal component of tissue repair and represents a dynamic and interrelated process comprising angiogenesis, tissue remodeling, inflammation and fibroblast contraction[144]. A proper balance between synthesis and degradation of extracellular matrix

molecules (e.g. collagen) is also of critical importance for the outcome of this process. After initial injury, cytokines are generated, which are important in the functional restoration of damaged tissue. Prolonged production of these cytokines, however, can lead to excessive matrix accumulation and chronic fibrosis (*Figures 15 and 16*). The consequences of such fibrosis, as found in desmoplastic lesions and in association with some tumors, represent a neoplastic manifestation of disordered repair and are often associated with serious clinical sequelae such as obstruction, ischemia, and disfigurement. Carcinoid fibrosis is as such an unregulated, fibrotic neoplastic process for which the biological basis has not been defined.

Carcinoid Fibrosis-Related Factors

A number of different factors potentially involved in small bowel carcinoid fibrosis have been examined. The focus has largely revolved around the secretory products of the small bowel-derived enterochromaffin (EC) cell.

Serotonin

The relationship between serotonin and small bowel fibrosis is not well characterized and data regarding its role are often inconsistent. In 1994, Moertel hypothesized based on his clinical data that serotonin may play a vasoconstrictive role in ischemia[145]. Methysergide and ergotamine are serotonin antagonists that are used in the treatment of migraine headaches and an uncommon side-effect of their usage is retroperitoneal fibrosis[146]. It was initially thought that these anti-serotonergic agents may cause fibrosis by interacting with serotonin receptors in some "unknown" fashion[147]. This was a surprising concept given the hypothesis that serotonin itself stimulated fibrosis presumeably via activation of a serotonin receptor. The specificity or validity of this observation, however, appears to be low given the

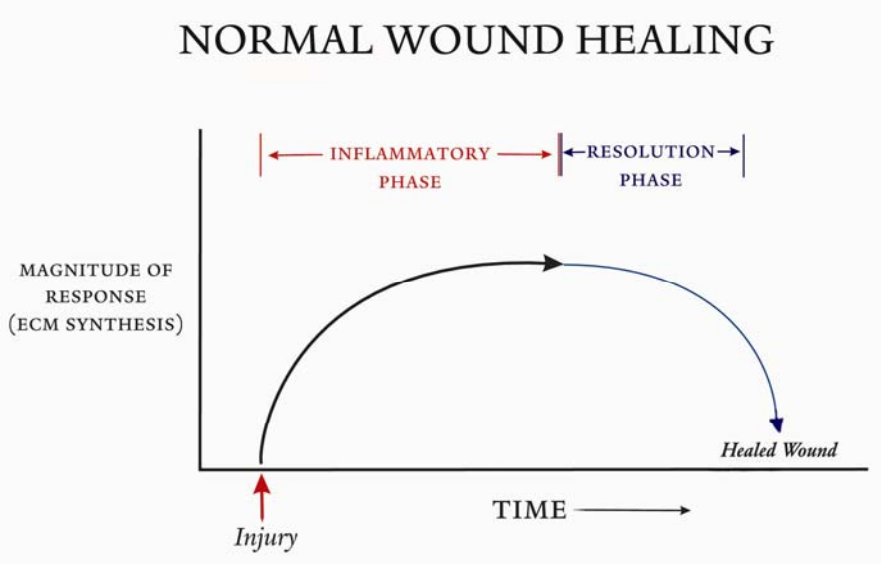


***Figure 15**: A proper balance between synthesis and degradation of extracellular matrix molecules (collagen) after cellular injury characterizes the normal wound healing process.*

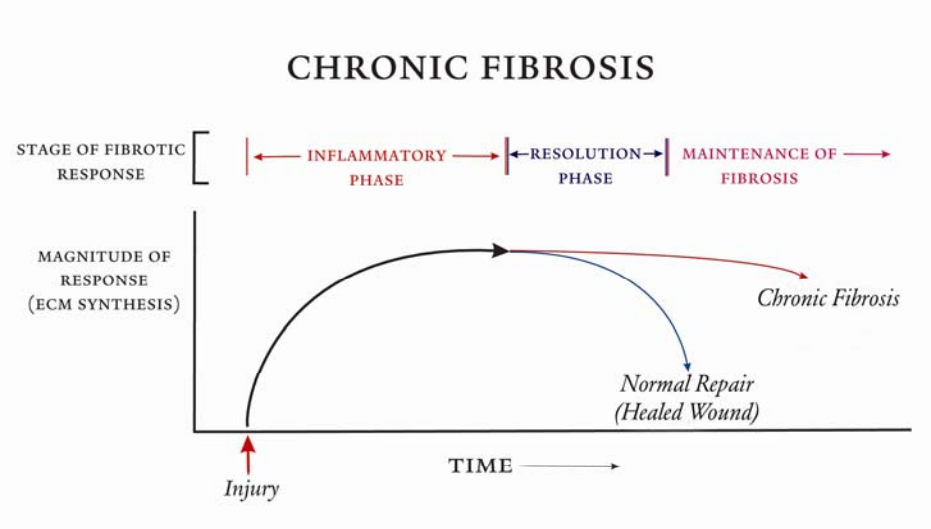


***Figure 16**: Fibrosis is usually a normal component of tissue repair and represents a dynamic and interrelated process comprising angiogenesis, tissue remodeling, inflammation and fibroblast contraction. When this process goes awry, it can lead to excessive production and deposition of collagen and chronic fibrosis. Although fibrotic disorders can be acute or chronic, the disorders share a common characteristic of excessive collagen accumulation and an associated loss of function when normal tissue is replaced with scar tissue.*

fact that other serotonin antagonists that interact with the same receptor (e.g. cyproheptadine and pizotifen, both of which are used to treat migraines) are not associated with fibroblastic

responses[148]. It is more likely that the antagonist, by interacting with a low-affinity (non-specific) serotonin receptor, may ameliorate or incite an inhibitory effect on fibrosis.

Whatever the mechanism, the finding that a serotonin antagonist causes fibrosis strongly supports that other factors are important in this process and that the implication of serotonin itself is likely to be an upstream event or a correlatable epiphenomenon given its secretion by the EC cell. More substantial evidence, however, exists for serotonin as an etiologic agent in the development of carcinoid heart disease. Several studies have demonstrated that patients with carcinoid tumors, those with cardiac involvement have higher levels of 5-HIAA, than patients without cardiac involvement[149-153].

One recent study[90] has demonstrated a close relationship between serotonin and cardiac disease. Support for a role for serotonin in valvulopathy has been described after prolonged administration of the diet drug combination of flenfluramine-phentermine[154]. Of note, the anti-migraine drug methysergide (associated with retroperitoneal fibrosis) is also associated with heart valve fibrosis[146], suggesting that a similar process may occur in this tissue. Because methysergide cannot be considered a selective antagonist, other explanations including concomitant adrenergic blockade, CNS-mediated hypotensive effects, and even a partial agonist activity have been proposed to explain this fibrogenic effect. Serotonin, however, itself is mitogenic to fibroblasts in culture[155], but only under circumstances where such cells have been transfected with the appropriate receptor[156,157]. Attempts to induce cardiac lesions of the carcinoid syndrome by chronically injecting pharmacological amounts of serotonin in animals have been unsuccessful[158]. The concept that serotonin plays a role in fibrosis is therefore not based on conclusive evidence and definitive studies have yet to be performed. It appears most likely, given the balance of data and the clinical observations with

respect to the retroperitoneum, that while an absolute increase in serotonin appears to be significant in relationship to the development of fibrosis, this may represent a correlatable epiphenomenon. It is more likely that secreted factors other than this agent play important roles in the development of fibrotic lesions.

Tachykinins

The role of tachykinins in fibrosis may be important, as members of this family (e.g. neurokinin A, substance P) have been demonstrated to stimulate fibroblast growth[159], and the same tachykinins are known to be secreted in a majority of patients with midgut carcinoids[3]. Very little, however, is known about these mediators and their relationship to carcinoid fibrosis, as there is a paucity of studies investigating this subject.

Growth Factors

In the last two decades, focus shifted from serotonin to growth factors as the etiologic agents of carcinoid-related fibrosis. Growth factors represent a heterogeneous group of polypeptides that act locally and stimulate cell proliferation and differentiation by binding to specific high-affinity cell membrane receptors[160,161]. Such polypeptides stimulate cell proliferation by diffusing short range through intracellular spaces and acting locally, contrary to the more distant endocrine action of hormones. Expressed in various mammalian cells and tissues, growth factors have been noted to play an increasingly significant role in development, wound healing, and carcinogenesis. Recognition of the mitogenic properties of growth factors on fibroblasts became evident and a putative role for their involvement in carcinoid tumor growth became apparent. Candidates included platelet-derived growth factor (PDGF), insulin-like growth factors I and II (IGF-I and -II), epidermal growth factor (EGF),

and the tissue growth factor-alpha and -beta (TGF-α and TGF-β) families of peptides, all of which have been demonstrated in malignant cells[162]. Proliferation and differentiation of cells in normal tissues is tightly controlled by several growth factors, and perturbations to this process may lead to uncontrolled growth and tumor formation[160].

PDGF

PDGF may play a role in connective tissue cell proliferation during chronic inflammation. The PDGF-β receptor, not typically expressed on resting cells in normal tissues, has been reported to be induced on connective tissue cells in chronic inflammatory conditions such as rheumatoid arthritis and rejected kidney transplants[163]. Chaudhry demonstrated that fibroblasts in carcinoid tumors express multiple PDGF receptors, suggesting that they respond to any of the three dimeric forms of PDGF[164]. Interestingly, the surrounding stromal component of these tumors synthesizes PDGF-α and -β chains, which may stimulate the growth of carcinoid tumor cells in a paracrine manner[164]. In a series of 31 midgut carcinoid tumors, the PDGF-β receptor was demonstrated by immunohistochemical staining in 66% of tumors (compared to only 9% of non-neuroendocrine tumors staining positively for the same receptor), and the stromal cells adjacent to the tumor cells stained more strongly than stromal cells that were distant from tumor clusters[163]. This suggests that carcinoid tumor cells may directly or indirectly induce expression of the PDGF-β receptor on adjacent stromal cells in the tumor tissue, which may possibly contribute to the stimulation of connective tissue cell proliferation in carcinoid tumors[163].

IGF-I

IGF-I, structurally homologous with proinsulin and biologically similar to insulin, plays an

important role in the physiological regulation of cell growth and differentiation[165]. The mitogenic effects of IGF are mediated via the IGF-I and -II receptors. Nilsson and his colleagues demonstrated the presence of the IGF-I receptor in 11 consecutive cases of midgut carcinoid tumors, suggesting that it may possibly act as an autocrine stimulator of carcinoid tumor growth[162].

EGF and TGF-α

Epidermal growth factor (EGF), in addition to the TGF-α and -β families of growth peptides, can stimulate or inhibit proliferation and differentiation in multiple tissues[166]. TGF-α, a peptide structurally related to epidermal growth factor (EGF), is expressed in abnormally high quantities in tumor cells and mediates its effects by binding to the EGF receptor[162]. In a series of 18 midgut carcinoid tumors, TGF-α and the EGF receptor were expressed in every specimen, suggesting that TGF-**α** participates in the autocrine modulation of carcinoid growth[162].

TGF-β

The TGF-β family of growth factors, neither structurally nor functionally related to TGF-α, exists in three isoforms (β1, β2, β3) in mammalian cells and tissues and acts as a potent autocrine growth-inhibitory factor in normal cells[160,167]. TGF-β has been found in most fibrotic lesions and is considered to be a critical profibrotic mediator due to its known ability to stimulate collagen synthesis[168]. TGFβ1 and the receptor subtype-2 (TGFβrII) have been identified in midgut tumor samples[169,170]. Similarly it has been demonstrated that tumor cells from carcinoids express TGFβ1 while stromal cells express the TGF-βII receptor[171]. This suggests that TGFβ1 may play an important role in the interaction between tumor and stromal cells. In a study that examined patients undergoing valve replacement surgery[172],

TGF-β was detected in the fibroblasts of all analyzed heart plaques. This supports the concept of an induction of TGF-β during the development of the fibroproliferative lesions in carcinoid heart disease. While one study demonstrated that serotonin may up-regulate TGF-β1 in aortic valve interstitial cells[173], a second study in a small number of patients found no relationship between circulating levels of TGF-β and carcinoid heart disease[151]. Despite this latter finding, a current hypothesis is that serotonin may regulate TGF-β-mediated fibrosis in this disease process[173].

A summary of the proposed biological mechanisms of the growth factors associated with carcinoid tumor growth is illustrated in *Figure 17*.

Connective Tissue Growth Factor

Recent studies have investigated the role of connective tissue growth factor (CTGF), a novel, cysteine-rich peptide involved in the coordination of complex biologic processes such as differentiation and tissue repair[174]. CTGF is the prototypic member of the CCN family of proteins[175,176], which are 30–40 kDa cysteine-rich proteins (*Figure 18*). Discovered in the early 1990's, these proteins stimulate mitosis, adhesion, apoptosis, extracellular matrix (ECM) production, growth arrest and migration. CTGF has been detected in fibroblasts, cartilaginous cells and chondrocytes, cancer cell lines, smooth muscle cells, renal podocytes and mesangial cells, hepatic sinusoidal cells, myofibroblasts, pancreatic acinar and ductile cells, bronchoalveolar ductile cells, as well as in serum, tear fluid, and urine[177]. CTGF participates in several physiological processes, including embryonic development and differentiation, endochondral ossification, wound healing, and angiogenesis[178]. Depending on the cell type, CTGF has been noted to promote mitosis, chemotaxis, stimulate apoptosis,

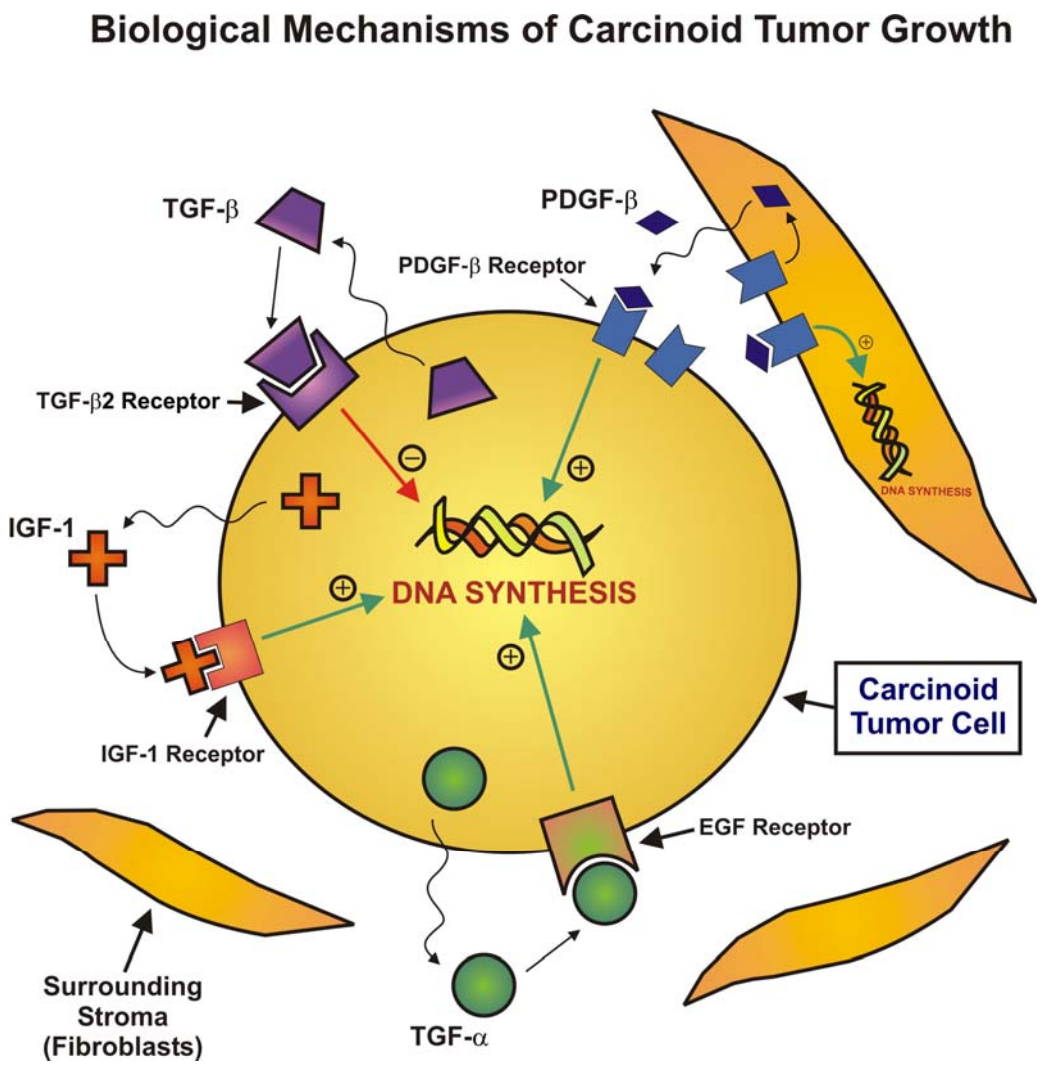


***Figure 17**: Proposed mechanism of the potential effects of small bowel carcinoid tumor-derived CTGF on local (retroperitoneal) and distant (cardiac) fibrosis. TGFβ1 auto-activates CTGF mRNA synthesis and protein release from the small bowel carcinoid tumor cells. Secreted CTGF and TGFβ1 interact locally with stromal cells, inducing collagen synthesis and deposition (and the resultant fibrosis in 42-78% of patients) in the retroperitoneum. CTGF also enters the bloodstream and may potentially act distantly in the heart via activation of cardiac fibroblasts with collagen deposition and the consequent endocardial fibrosis, valvular fibrosis, and right-sided heart failure (noted in 20-38% of patients).*

angiogenesis, synthesis of collagens, fibronectin and α5-integrin[179]. The low density lipoprotein (LDL) receptor-related protein/α-2-macroglobulin receptor (LRP2) has been demonstrated to be the receptor for CTGF in fibroblasts[180]. By signaling via integrins, a mechanistic interpretation is provided for the chemotactic and mitogenic properties of CTGF,

as well as for its functions in ECM remodeling during angiogenesis and wound healing[177].

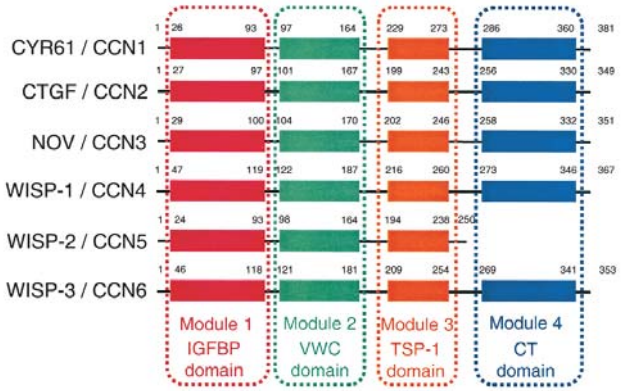


Figure 18: *Schematic representation of the CCN family of matricellular proteins that have been grouped together based on a similar predicted structure. CTGF (CCN2) plays an essential role in the formation of blood vessels, bone, and connective tissue.*

Several factors are capable of regulating CTGF gene expression, although it is generally believed that CTGF is transcriptionally activated primarily through TGFβ1. In addition to TGFβ1, CTGF gene expression is also influenced by elevated glucose levels, tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), cortisol, cAMP, coagulation protease thrombin, prostaglandin E2 (PGE_2), drugs such as iloprost and statins, as well as with cytomegalovirus infection[181-189]. Of particular relevance, however, are recent reports that describe how other growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF), activate CTGF gene expression at a transcriptional level[178,190].

The intracellular pathways regulating CTGF are still not clear. Recently, Leask and his colleagues reported that SMAD, PKC and ras/MEK/ERK (kinase) pathways are necessary for the TGFβ1-mediated induction of the CTGF promoter[191]. As a mediator of fibrosis, the expression of CTGF has been noted to increase in several pathological conditions involving inflammation and connective tissue accumulation. The expression of CTGF has been reported to be practically absent in normal human arteries, but highly enhanced in the intimal smooth muscle cells of atherosclerotic lesions and in myocardial infarctions[192]. In the human

kidney, the expression of CTGF has been found to be markedly increased in glomerular and interstitial inflammatory lesions with cellular proliferation and matrix accumulation (e.g. IgA nephropathy, chronic transplant rejection, crescentic glomerulonephritis, focal glomerulosclerosis, lupus nephritis and membranoproliferative glomerulonephritis)[193]. Furthermore, the up-regulation of CTGF has been noted in numerous other pathological conditions, including scleroderma, keloid formation, malignant melanoma, chronic pancreatitis, hepatitis and liver cirrhosis, Crohn's disease, ulcerative colitis, pulmonary fibrosis, and sarcoidosis[179].

CTGF mediates normal scar formation in wound healing[174]. Physiologically, the activity of TGFβ1 and CTGF diminishes as the wound heals and adequate scar formation is achieved[177]. However, in pathological diseases where the inflammatory component remains, the expression of CTGF stays elevated with a concomitant elevation in ECM production[177]. The chronic induction of the CTGF expression results in a pathologic fibrosis of the involved organ and development of abnormal fibrotic tissue[177].

HYPOTHESIS

CTGF is a substantial candidate for consideration as the local and circulating regulator of small bowel carcinoid fibrosis. I hypothesize that the ill-understood and dangerous phenomenon of fibrosis associated with small bowel carcinoids is specifically due to a secreted tumor factor derived from the EC cell, namely CTGF. The overall aims of this study are to address whether CTGF is over-expressed and secreted by small bowel EC carcinoid tumors, determine if there exists a relationship between CTGF and small bowel carcinoid fibrosis, and if so, what role this growth factor plays in the genesis of ileal carcinoid-related fibrosis and whether measuring serum levels of CTGF has any clinical utility in the identification of patients with carcinoid tumors and/or fibrosis. Schematic models of this hypothesis (*Figures 19 and 20*) illustrate the proposed profibrotic effects of CTGF in patients with carcinoid tumors.

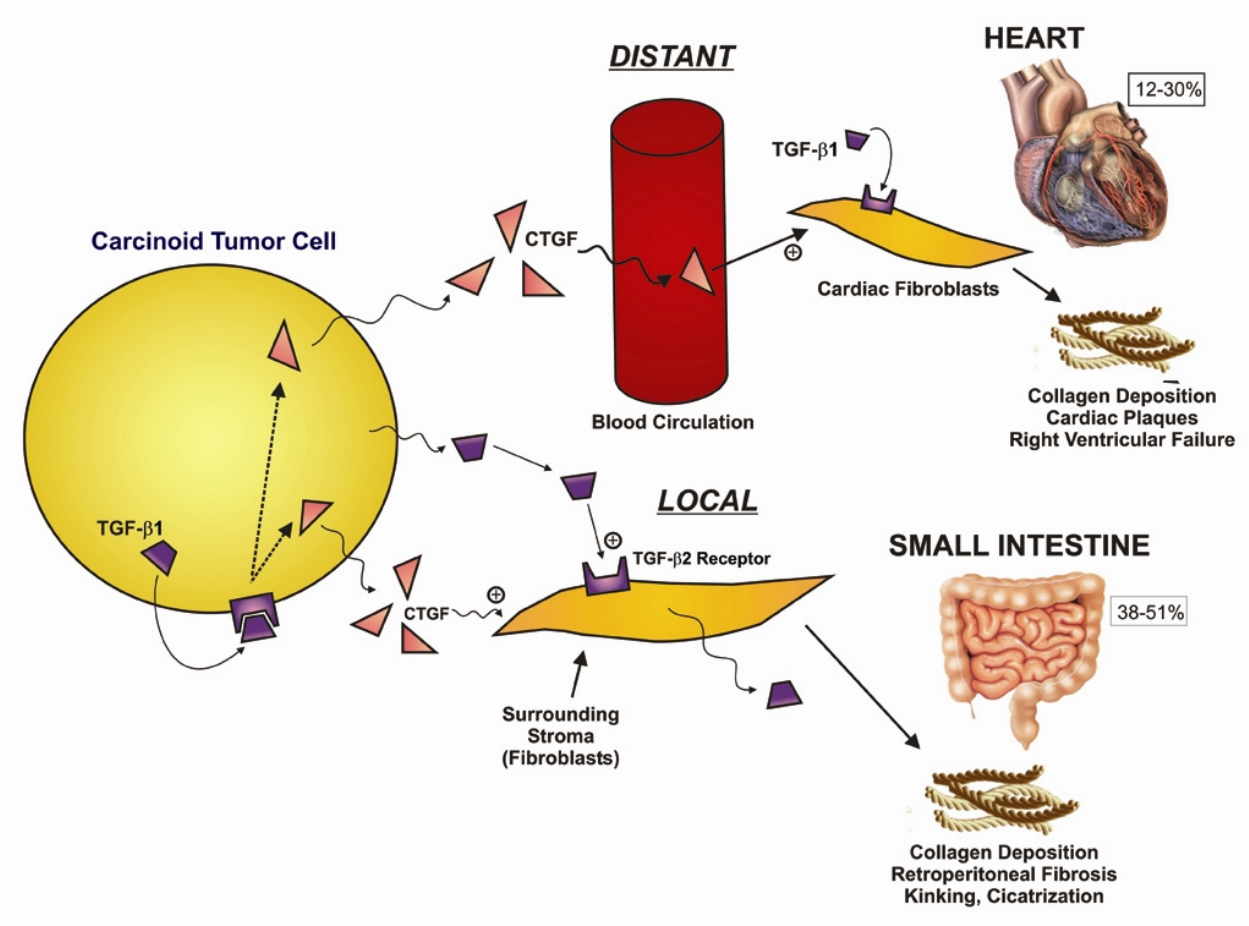


Figure 19: *Proposed mechanism of the potential effects of small bowel carcinoid tumor-derived CTGF on local (retroperitoneal) and distant (cardiac) fibrosis. TGFβ1 auto-activates CTGF mRNA synthesis and protein release from the small bowel carcinoid tumor cells. Secreted CTGF and TGFβ1 interact locally with stromal cells, inducing collagen synthesis and deposition (and the resultant fibrosis in 42-78% of patients) in the retroperitoneum. CTGF also enters the bloodstream and may potentially act distantly in the heart via activation of cardiac fibroblasts with collagen deposition and the consequent endocardial fibrosis, valvular fibrosis, and right-sided heart failure (noted in 20-38% of patients).*

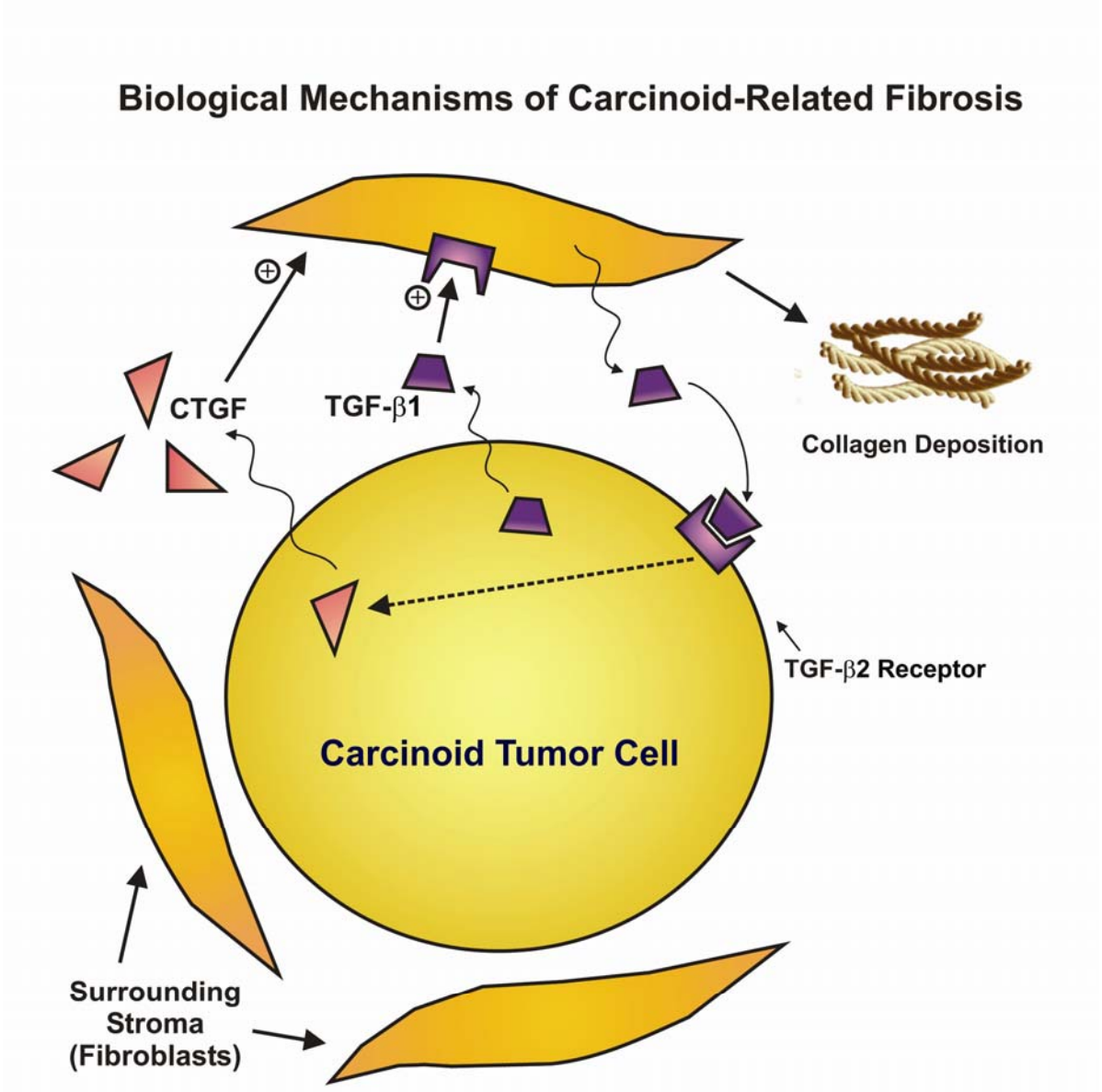


Figure 20: *Schematic representation of the proposed biological mechanisms responsible for carcinoid-related fibrosis. TGFβ1, released by both carcinoid tumor and stromal cells, interacts with the TGFβ receptor subtype-2 on the tumor cell, whereby its activation results in CTGF mRNA synthesis and protein release from the tumor. CTGF and TGFβ1 have been shown to be secreted in excess from EC carcinoid tumor cells. Once secreted, both TGFβ1 and CTGF interact with the stromal cells, inducing collagen synthesis and deposition (and the resultant fibrosis).*

STUDY DESIGN AND METHODS*

SPECIFIC AIM #1: EXAMINE SERA AND TISSUE SAMPLES USING STANDARD ASSAYS TO IDENTIFY WHETHER CTGF IS OVER-EXPRESSED AND/OR SECRETED BY SMALL BOWEL CARCINOIDS.

Serum and Tissue Sample Acquisition

Patient Recruitment: The P.I. (Dr. Irvin Modlin) is responsible for a neuroendocrine referral center at Yale University in consultation with Dr. John Murren and sees an average of 2–3 new patients with carcinoid disease on a weekly basis. I prospectively acquired sera from patients attending this clinic. A second patient resource is the Yale Surgical Oncology Group that examines patients with EC carcinoids or their liver metastases who are operated on each year, and there is a subset of these patients (7-10) who have carcinoid heart disease and are regularly examined. Additionally, a retrospective databank of 23 serum samples had been collected previously from patients attending the aforementioned clinics. These samples included 20 patients with GI carcinoids: small bowel EC carcinoids (*n=10*), gastric ECL cell carcinoids (*n=4*); and six other GI carcinoids (rectal: *n=2*, parotid: *n=1*, appendiceal: *n=2*, duodenal: *n=1*). Serum samples from three patients undergoing hernia repair were also collected. Tumor tissue from nineteen patients with gastrointestinal carcinoids (EC, $n = 16$; ECL, $n = 3$) as well as tissue from nine adjacent, unaffected areas had previously been collected as well. It was estimated that approximately 60 patients would be recruited per year from the following groups attending these clinics:

1. patients with small bowel carcinoids
2. patients with gastric carcinoids

3. patients with other diseases requiring surgery (e.g. ulcerative colitis, colorectal cancer, hernia repair)
4. normal subjects

Based on previous studies that demonstrate fibrotic complications in 16-48% of patients[4], I estimated that a minimum of 10 patients from the small bowel tumor group would have or develop fibrotic complications.

The recruitment and consenting of research subjects in this particular study was approved by the Human Investigations Committee at the Yale University School of Medicine (HIC #12589). Patients were required to sign an informed consent form that provided a brief description of the research project, the procedures used, risks and inconveniences, benefits, and economic considerations. All participants were informed of the voluntary nature of their participation and assured of the strict maintenance of confidentiality. Participants had the opportunity to ask any questions and were offered a copy of the information sheet for their records, which included contact information of the principle investigator. Upon signing and dating the consent form, blood samples were collected immediately.

Epidemiological characteristics were recorded for each patient, including age, sex, diagnosis, surgical history, radiological studies (particularly Octreoscan), pertinent medications related to their carcinoid disease (e.g. octreotide), and current therapeutic management of their carcinoid disease. A current or previous history of carcinoid syndrome was assessed by asking patients whether they have experienced several consecutive days of frequent episodes (>3/day) of diarrhea or facial flushing.

Tissue Collection

Our laboratory has established a frozen-tissue databank of tumors including enterochromaffin cell (EC) and enterochromaffin cell-like (ECL) carcinoids of the ileum and stomach, pancreatic gastrinomas, and adenocarcinomas of other gastrointestinal sites, as well as normal tissue sampled from sites adjacent to these tumors. This databank was augmented by tissue samples I collected prospectively during the experiment period.

As part of our approved HIC protocols (#12589 and #11041), I collected tissue samples in the operating rooms at Yale–New Haven Hospital from patients undergoing surgical resection of carcinoid tumors. As with the serum collection, informed patient consent was obtained pre-operatively for the collection and use tissue samples. In patients undergoing bowel resection or gastrectomy, tumor tissue was collected and paired with adjacent, normal mucosa. Each tissue sample was stored immediately in a -80°C freezer until needed.

Examination of CTGF Expression in Carcinoid and Normal Adjacent Tissues

The following RNA isolation and RT-PCR experiments were performed by Dr. Mark Kidd.

RNA Isolation: Total RNA was isolated from frozen carcinoid tumor tissue (n=19) and normal mucosa (n=9) using TRIzol reagent (Invitrogen, Carlsbad, CA) using the manufacturer's guidelines. RNA was dissolved in DEPC water, measured spectrophotometrically and an aliquot analyzed on a denaturing gel using electrophoresis to check the quality of RNA isolated.

RT-PCR: 19 tumor samples and 9 normal mucosal samples were examined by RT-PCR. 2 μg of total RNA underwent cDNA synthesis using SUPERSCRIPT Reverse Transcription (Invitrogen). For semi-quantitative PCR, GAPDH was used as a "house-keeping" gene (primers: Forward 5'-GTG AAG GTC GGA GTC AAC, Reverse 5'-GGT GAA GAC GCC

AGT GGA CTC). Thereafter, PCR was undertaken with each sample for CTGF (primers: Forward 5'-GAG GAA AAC ATT AAG AAG GGC AAA, Reverse 5'-CGG GAC AGG TCT TGA TGA)[194], and imaging densitometry undertaken (*NIH Image 1.6*, NIH, Bethesda) of the ethidium bromide gels to quantify the gene product.[195]

Quantitative RT-PCR Analysis of CTGF and TGFβ1 mRNA Expression in Small Bowel and Gastric Carcinoid Tumors

RNA samples were quantitatively measured for TGFβ1, TGFβRI, TGFβRII and $p21^{(WAF/CIP1)}$ message by Q-PCR as described[196]. Briefly, Q RT-PCR (was performed using the ABI 7900 Sequence Detection System. Total RNA from each sample was subjected to reverse transcription using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturers suggestions. Total RNA (2 μg/50 μl) was reverse-transcribed and real time PCR analysis was performed in duplicate on these samples. Briefly, cDNA in 7.2 μl of water was mixed with 0.8 μl of 20x Assays on Demand primer (TGFβ1= Hs00171257, TGFβRI=Hs00610319, TGF-βRII=Hs00559661, $p21^{(WAF/CIP1)}$= Hs00355782, GAPDH=Hs99999905) and probe mix, 8 μl of 2x TaqMan Universal PCR Master mix in a 384 well optical reaction plate. The following PCR conditions were used: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C/0.15 min and 60°C /1 min. A standard curve was generated for each gene using cDNA obtained by pooling equal amounts from each sample (n=12). The expression level of target genes was normalized to internal GAPDH. Data was analyzed using Microsoft Excel and calculated using the relative standard curve method (ABI, User Bulletin #2).

SPECIFIC AIM #2: DETERMINE IF THERE EXISTS A RELATIONSHIP BETWEEN CTGF AND SMALL BOWEL CARCINOID FIBROSIS BY GENETICALLY AND PROTEOMICALLY DEFINING THE SMALL BOWEL TUMOR FIBROSIS PHENOTYPE AS OPPOSED TO THE NON-FIBROGENIC GASTRIC CARCINOID TUMOR AND CORRELATE THIS INFORMATION WITH CLINICAL OUTCOMES FOR EACH PATIENT.

Clinical Relevance of Proteomics and Tissue Microarrays (TMA)

Detailed analysis of gene expression in both prokaryotes and eukaryotes using DNA microarray technology allows for parallel analyses of the expression of thousands of genes to address complex biological questions[197]. This is important because the properties of cancer cells can vary enormously from one patient to another; as such, it is often not possible to characterize individual tumors by means of a single, or even several, molecular markers. The properties of cancer cells reflect the functions of all their gene products. Presumably this is true for fibrosis as well. The clinical use of gene expression profiles has the potential of offering more accurate and objective diagnoses of cancers as well as prognoses of a disease or response to treatment.

GeneChip® microarray studies have provided a broad and efficient approach toward identifying such candidate markers and drug targets[198]. Gene expression analyses do not, however, provide reliable information on the actual proteins encoded by genes showing altered expression, because there is often no direct relationship between *in vivo* concentration of an mRNA and its encoded protein. Differential rates of translation of mRNAs into protein and differential rates of protein degradation *in vivo* are two factors that confound the extrapolation of mRNA to protein expression profiles[199]. While DNA arrays allow expression analysis of thousands of genes in a single specimen, the recently developed methods of

proteomics and tissue microarrays (TMA) provides a means to examine the protein expression of the genes.

Proteomics, or peptide profiling, is a relatively new field that has begun to show promise in the diagnosis, early detection and the monitoring of prognosis in many different diseases[200]. Differential two-dimensional fluorescence gel electrophoresis (DIGE) utilizes *in vitro* labeling with Cy3/Cy5 fluorophores, can detect a number of post-translational modifications, has an approximate dynamic range of 10,000; quantifies approximately 1,000 spots; and is optimized for naturally-occurring forms of proteins larger than 10 kDa[201]. This method is well-suited for the identification of proteins in specimens.

Proteomics has emerged as a useful tool for studying connective tissue biology[202]. In this study, we used this approach to determine the protein profiles of small bowel carcinoid tumors. TMA permits rapid molecular profiling of hundreds of pathological specimens[203] (high through-put analysis) and is therefore also suited for analyses of candidate proteins identified in gene expression and microarray experiments[197,204]. Formalin-fixed archival tissues provide a means to validate targeted gene-protein identification and genomic screening in large sets of histologically well-characterized samples with clinical endpoints.

To get a fuller picture of the complexity of processes and pathways involved in the pathophysiology of small bowel carcinoid fibrosis, efficient protein profiling and TMA immunohistochemistry is necessary to compliment the RNA analyses and provides additional insight into how genes act and interact at the protein level. We have begun to use a combination of gene, protein and TMA analysis in small bowel carcinoids, and this triple approach is being used to identify how fibrosis is generated and for the identification of small bowel fibrotic mediators.

Carcinoid Tumor TMA: Design and Applications

With assistance from Dr. Robert Camp of the Yale University Department of Pathology Tissue Microarray Facility, a carcinoid tissue microarray was constructed from banked pathology specimen blocks. This microarray (YTMA#32; the only such carcinoid-specific array in the United States) includes thirty-six patients identified at Yale University over the last twenty-four years with carcinoids of the small bowel (no fibrosis: *n=21*; fibrosis: *n=15*), 7 patients with gastric carcinoids, normal liver and normal bowel tissue. A second array (YTMA#60) is presently being constructed with the tissue samples I obtained over the course of this study, which includes fibrotic, metastatic, normal mucosa and cardiac tissue. Currently, our collection includes 55 small bowel carcinoids, 12 gastric carcinoids, metastatic tissue in the lymph nodes (*n=14*), metastatic tissue in the liver (*n=10*), normal adjacent mucosa from every tumor (*n=67*), and fibrosis (*n=28*) from small bowel carcinoids.

The tissue microarray is a novel method of harvesting small cylinders of tissue from standard histological sections and placing them in a well-defined matrix in a recipient paraffin block[205] (*Figure 21*). Sections are then made of this multi-sample block and affixed to standard glass microscopy slides, which can then be used for simultaneous *in situ* analysis of primary tumors, material of interest (e.g. areas of fibrosis), or non-affected tissue at a protein level. The large number of tissue samples on a single slide can be simultaneously immunostained using this technique,

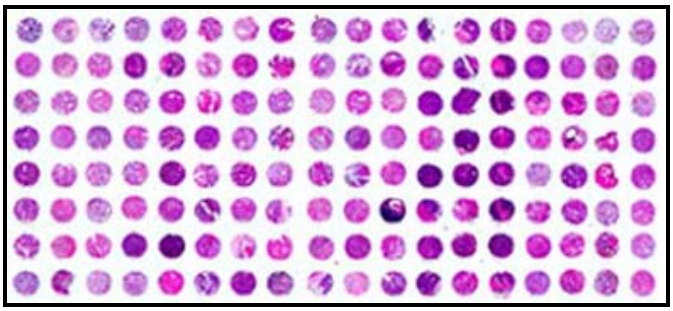


Figure 21: *Example of a tissue microarray. With this technique, hundreds of tissue samples can be studied simultaneously.*

and a variety of clinical parameters (e.g. presence or absence of fibrosis, dissemination of disease, patient survival characteristics, operative course) can then be correlated with these results in a single experiment (*Figure 22*)[206].

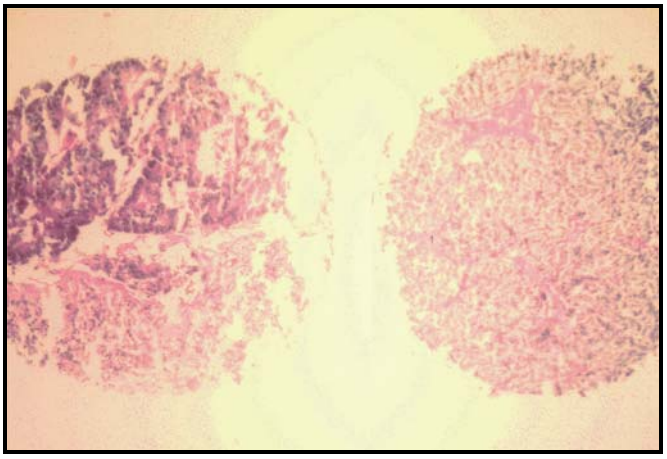


Figure 22: *H&E stain of the carcinoid tissue microarray showing two tumor sections on the grid (100x magnification).*

Construction and Processing of GI Carcinoid TMA

Formalin-fixed paraffin-embedded tissue blocks containing GI carcinoids (stomach: *n=7*; duodenum: *n=5*; small bowel: *n=36*; appendix: *n=20*; colon: *n=7*) were retrieved, along with the corresponding H&E-stained slides, from the archives of the Yale University School of Medicine Department of Pathology prior to the start of this project. Blocks were stored under ambient conditions within a temperature range of 18-37°C. To ensure uniformity of sectioning, older paraffin blocks were melted and re-embedded using modern-day plastic cassettes. Tumors were staged and histologically typed according to the recommendations of the pathologist (Dr. Robert Camp). Normal and areas of fibrosis were appropriately identified. A full-time lab technician constructed the TMA by taking core tissue biopsies 0.6 mm in diameter from carefully selected morphologically representative regions of individual paraffin-embedded tumors, and/or adjacent fibrosis and/or normal mucosa and precisely arrayed into a new recipient paraffin block (45 mm x 20 mm) using a custom-built instrument. Sections of the resulting tissue microarray block 5 μm thick are then transferred to glass slides by the technician using the paraffin sectioning aid system (adhesive coated

slides PSA-CS4x, adhesive tape, UV lamp, Instrumedics Inc., Hackensack, NJ) to support the cohesion of 0.6-mm array elements. Immunohistochemical analysis could then be performed on proteins of interest (e.g. CTGF).

Two separate tissue microarrays were used in this study. The first array contained 10 cases and was used to titrate antibodies and establish the efficacy of the array. The seconds array contained 75 cases of primary GI carcinoids diagnosed between 1965 and 2001 and was represented by 2 cores per case. Follow-up information was available (median follow-up: 9 years, range: 2-38 years) for all patients. This microarray includes thirty-six patients with carcinoids of the small bowel in whom the fibrotic and metastatic/malignant-associated clinical details were known.

Immunohistochemical Staining of TMA

Tissue microarray slides were stained as described by Dr. Camp and his colleagues[206]. In brief, for automated analysis, slides are incubated for 1 hr at room temperature with mono- or polyclonal antibodies (1:200-1:1,000; Santa Cruz Biotechnology Inc, CA or DAKO Corp, Carpinteria, CA, or Zymed Laboratories, San Francisco, CA) diluted in Tris-buffered saline containing BSA. Secondary antibodies conjugated to a horseradish peroxidase-decorated dextran polymer backbone (Envision; DAKO Corp) were used. For automated analysis, tumor cells were identified by the use of a fluorescently tagged anticytokeratin antibody cocktail (AE1/AE3; DAKO Corp), after which 4',6-diamidino-2-phenylindole was added to visualize nuclei, and proteins of interest were then visualized with a fluorescent chromogen (Cy-5-tyramide; NEN Life Science Products, Boston, MA). Cy-5 (red) was used because its emission peak is well outside the green-orange spectrum of tissue

autofluorescence. Protein expression was determined using an automated tissue microarray reader controlled by Dr. Camp.

Automated image acquisition and analysis using AQUA has been described previously by Dr. Camp and his colleagues as well[206]. In brief, monochromatic, high-resolution (1024 x 1024 pixel; 0.5-μm) images are obtained of each histospot. Areas of tumor can be distinguished from stromal elements by creating a mask from the cytokeratin signal. Coalescence of cytokeratin at the cell surface helps to localize the cell membranes, and 4',6-diamidino-2-phenylindole is used to identify nuclei as mentioned above. The red Cy-5 signal from the membrane area of tumor cells is scored on a scale of 0–255 and expressed as signal intensity divided by the membrane area. Histospots containing <10% tumor, as assessed by mask area (automated), are excluded from further analysis. Previous studies have demonstrated that the staining from a single histospot provides a sufficiently representative sample for analysis[207]. Previous data have also demonstrated that mean alterations in AQUA scores of 7 (in a range of 0-255) are sufficiently sensitive to discriminate protein expression and provide statistically significant data[206]. In addition, a minimum number of samples (n=10) is required for statistical significance[204].

Dr. Kidd and I deparaffinized consecutive sections of the TMA or normal small bowel mucosa in xylenes and rehydrated them in graded alcohols. Antigen retrieval and immunostaining was performed as described above. Serial sections (5 μm) were incubated with goat antiserum to CTGF (1:250) and a monoclonal antibody against CgA (0.5 μg/ml) or serotonin (2 μl/ml) (both from DAKO) for 24 hr at 4°C and then with FITC-labeled anti-rabbit and TRITC-labeled anti-goat IgG (1:200 dilution) for 1 hr at RT. HRP-amplification was performed as above and bound antibodies visualized under light microscopy. Single-

positive (either serotonin or CgA) and dual-positive cells (CTGF + serotonin or CTGF + CgA) were counted in a minimum of 5 well-orientated crypts and expressed as a percentage.

The unpaired 2-tailed Student's *t*-test was used to identify statistically significant differences in fibrotic protein expression between different patient groups (fibrosis versus non-fibrosis, fibrosis versus gastric carcinoid). The significance was set to $p<0.05$. The χ^2-test (Fisher's exact text) was used to evaluate the statistical significance (presence or absence of staining) in any two groups.

SPECIFIC AIM #3: DETERMINE WHETHER MEASURING SERUM LEVELS OF CTGF HAS ANY CLINICAL UTILITY IN IDENTIFYING PATIENTS WITH FIBROSIS.

Serum Collection

After obtaining consent from the patient, I collected 10 ml of peripheral venous blood using a 21-gauge Vacutainer Safety-Lok Blood Collection Set (Becton Dickinson, Franklin Lakes, NJ) into two 6 ml lithium heparinized plastic Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). The samples were then centrifuged for five minutes at 1000g within thirty minutes of collection. The serum was aliquoted into 1.8 ml Nunc cryotubes (Nunc A/S, Roskilde, Denmark) and stored at -20° C until needed for various analytical assays.

Prior to analysis, the frozen samples were left to stand at room temperature to thaw, and then inverted several times to mix. The plasma aliquots from all temperature and time-points for each volunteer were analyzed together in one batch, to avoid run-to-run variability.

Serum Analysis

The first objective was to identify the serum secretory profile of CTGF secretion in carcinoid tumors. Because TGFβ1 is an upstream regulator of CTGF, this was measured as well, along with Chromogranin A and serotonin. Chromogranin A was used as a marker of tumor mass, and serotonin was examined because it has been identified as a marker for carcinoid heart disease [90]. Each serum sample was analyzed for these four proteins. Fasting samples were obtained to obviate any exogenous humoral release.

Serum CTGF levels

Based on our preliminary data (mean serum CTGF levels = 23 ng/mL in carcinoid patients vs. 12 ng/mL in patients with other tumors) and using a 99.5% significance level ($p < 0.005$ that the hypothesis is true due to chance alone), and a power of 1.0, the numbers of serum samples from patients to be prospectively examined for CTGF levels was calculated to be 15. The sera of a subgroup of these patients was examined serially to verify the inter- and intra-test reproducibility of the assay and to examine whether the serum levels of CTGF are constant or fluctuate as a function of time or surgical removal of a carcinoid tumor.

Temporal expression of serum CTGF as well as the effects of surgery on CTGF levels in a number of different groups of patients (hernia repair and tumor resections) was examined by measuring pre-operative baseline serum CTGF followed by another serum measurement 24 hour postoperatively.

Potential Confounders:

1) Pharmacotherapeutics: Because it was unknown whether Sandostatin® (a somatostatin analogue used in the treatment of carcinoid symptoms)[208] might affect either TGFβ1 or

CTGF secretion, it was treated as a potentially confounding variable. I therefore examined fasting serum levels in new patients to the clinic (who were not on the drug) and later during the course of their treatment at 3 monthly intervals (if and/or when they have begun treatment of this drug). For patients who were taking the long-acting regimen (LAR), serum was obtained immediately prior to their next injection. For patients who were injected with the short-acting somatostatin analogue, octreotide (which has a half-life of 8 hr), a 72 hr injection-free period was followed prior to serum acquisition.

2) Surgery: when serum samples were needed from patients undergoing hepatic embolization or surgical resection, I collected the samples immediately before the procedure.

CTGF Serum ELISA

Serum CTGF-W (whole molecule) and CTGF–N (N-terminal fragment) was measured by Dr. William Usinger of Fibrogen (at their lab facilities in San Francisco, CA) by sandwich enzyme-linked immunosorbent assay (ELISA) using two non-cross blocking proprietary monoclonal antibodies reacting to distinct NH_2-terminal epitopes of CTGF as described[209]. In brief, ELISA plates (Immulon 2) were coated overnight with a monoclonal antibody directed to the amino terminus of CTGF. Wells were washed and blocked with buffer containing BSA and then rinsed. Another amino-terminal anti-CTGF monoclonal antibody solution (50 μl) conjugated to biotin was added. Then 50 μl (in duplicate wells) of each standard, control, or serum sample, pre-diluted in assay buffer, was then added to the plate, covered, and incubated at 4°C on a plate shaker for 1.5 h. The plate was washed three times with wash buffer, and 50 μl of a solution of streptavidin conjugated to alkaline

phosphatase was added to each well. The plate was again covered and incubated at 4°C on a plate shaker for 1.5 h. The plate was washed three times with wash buffer. Then 100 μl of substrate buffer containing para-nitrophenyl phosphate was added to each well. After proper color development, the enzyme activity was stopped by the addition of 50 μl of 4 N NaOH. The plate was read at 405 nm, and data was fitted using a quadratic fit option. Standards were made from purified full-length CTGF and expressed in nanograms per milliliter. The described CTGF ELISA is capable of detecting both full-length CTGF (CTGF-W) as well as an amino-terminal fragment of CTGF. The intra- and interassay co-efficient of variations are 5 and 15%, respectively.

TGFβ1 serum ELISA

This assay, performed under the guidance of Dr. Kidd, employed the quantitative sandwich enzyme immunoassay technique as described.[210] Briefly, TGFβ soluble receptor type II, which binds TGFβ1, was pre-coated onto a microplate. Standards and samples were pipetted into the wells and any TGFβ1 present was bound by the immobilized receptor. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGFβ1 was added to the wells to sandwich the TGFβ1 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, I added a substrate solution to the wells and color developed in proportion to the amount of TGFβ1 bound in the initial step. The color development was then stopped and the intensity of the color measured. The intra-assay and inter-assay coefficients of reproducibility for these assays are 6% and 9% respectively.

Serotonin ELISA

I quantitatively measured serum serotonin levels by competitive enzyme-linked immunosorbent assay (IBL, Hamburg), whereby there is competition between a biotinylated and a non-biotinylated antigen for a fixed number of antibody binding sites. The amount of biotinylated antigenic bound to the antibody is inversely proportional is the analyte concentration of the sample. I performed this experiment under the supervision of Dr. Kidd.

All assay reagents were stored in the refrigerator and were allowed to reach room temperature before starting the experiment. 20 μl of serum was pipetted into glass test tubes. 100 μl of diluted Assay Buffer was added to each tube and vortex mixed. 25 μl of Acylation Reagent was then added to each tube, vortex mixed, sealed with Parafilm, and incubated for 15 minutes at 37° C. 4 ml of diluted Assay Buffer was added to each tube, vortex mixed, and the precipitated proteins were removed by centrifugation (10 minutes at 1500 RPM). 50 μl aliquots of supernatant were then withdrawn immediately from each sample. 15 μl of each standard, acylated control serum, and acylated patient samples were pipetted into the appropriate wells. 50 μl of serotonin biotin were pipetted into the wells. 50 μl of serotonin antiserum was then pipetted into the wells and the ELISA plate was shaken carefully. The plate was sealed with an adhesive foil and incubated 18 hours overnight at 4° C. Each well was washed three times with Wash Buffer. The plate was inverted and tapped firmly on clean blotting paper to remove any remaining liquid. 150 μl of enzyme conjugates were pipetted into each well. The plate was then sealed with a piece of

foil and incubated for 120 minutes at room temperature on an orbital shaker (500 RPM). Each well was washed three times with wash buffer, and 200 μl of PNPP Substrate Solution was then pipetted into each well. The plate was incubated at room temperature for 60 minutes on an orbital shaker (500 RPM). The substrate reaction was stopped by adding 50 μl of PNPP stop solution into all wells, and the contents were briefly mixed by gently shaking the plate. The plate was immediately read with a microtiter reader at an optical density of 405 nm and the data analyzed with the assistance of Dr. Kidd using a cubic spline function.

The results were calculated by subtracting the optical density of the substrate blank from the optical densities of all standards in samples. The sample preparation leads to a 207-fold dilution, and thus the values read from the standard curve were corrected by a multiplying each value by 207 to give the serotonin concentration in ng/ml. The intra- and interassay CVs were 7.2 and 10.5%, respectively.

Chromogranin A ELISA

I measured the serum CgA levels using a commercially available CgA ELISA kit (Dako A/S, Denmark). The kit utilizes an immunoenzymatic sandwich methodology. The kit can be used for measuring chromogranin A in the range 10 to 500 U/L, and the normal value is $\leq$17 U/L. The ELISA is a simplified double antibody sandwich assay where the sample and the conjugate were incubated simultaneously in antibody-coated wells, with results obtained in 2 ½ hours. The total imprecision of the assay is less than 9% over the entire measurable

range[211]. The coefficient of variation ranged from 10.2% (intra-assay) to 12.4% (inter-assay).

SPECIFIC AIM #4: DETERMINE IF THE IDENTIFICATION OF SPECIFIC FIBROTIC MEDIATORS WOULD BE HELPFUL IN SCREENING CANDIDATE FIBROSIS-ASSOCIATED GENES AND PROTEINS IN SMALL BOWEL CARCINOID TUMORS.

GeneChip® DNA Microarray Studies

A variety of serum markers have been proposed to detect and predict fibrotic disease, particularly hepatic fibrosis (most commonly in the context of hepatitis C infection) because of their proposed ability to identify patients with increased or progressive collagen synthesis[212]. The use of serum markers as direct biochemical markers of fibrosis has been evaluated in several studies. Among the more commonly investigated serum markers are alpha-2-macroglobulin[213], hyaluronic acid (HA)[214,215], YKL-40, gamma-glutamyl transpeptidase (GGT)[216], procollagen type III *N*-terminal peptide (PIIINP)[217,218], and matrix metalloproteinases (MMP-1, MMP-2, MMP-9)[219,220]. The prothrombin index and HA appear to be the best predictive factors for cirrhosis[221], while serum YKL-40 may be related to the degree of liver fibrosis, with the highest levels found in patients with severe or moderate cirrhosis and lowest in those without fibrosis[222]. The combination of two serum markers reflecting fibrogenesis (PIIINP) and fibrolysis (MMP-1) may provide a useful tool for evaluating liver fibrosis[220]. Commercially available serum kits, such as FibroTest® and ActiTest®, have been developed for comprehensive analysis of six serum markers simultaneously, but it is clear that these kits may not accurately predict the presence or absence of significant liver fibrosis[223]. The relevance of any of these markers to small bowel

carcinoid fibrosis has never been evaluated, and the clinical utility of these kits (which were developed for hepatic disease, as they measure liver markers of tissue damage) in detecting small bowel carcinoid fibrosis is not known. Because the biology of small bowel carcinoid fibrosis is poorly understood, it would be clinically useful to identify fibrotic mediators specific to this tissue and then determine the clinical utility of these organ-targeted candidate fibrotic proteins rather than using a hepatic-based test of undetermined relevance.

The tissue databank was used to identify the gene expression patterns of fibrotic small bowel carcinoid tumors using the established Affymetrix-based GeneChip® approach[224]. Three groups of tumors were examined - gastric carcinoid tumors and two groups of small bowel carcinoid tumors (invasive, non-fibrotic tumors and fibrotic tumors) and one control group (normal small bowel mucosa).

Two gene analysis approaches were used. In the first, whole tissue sections from carcinoid tumors were used. This allowed for the identification of both tumor and surrounding tissue genes, providing an overall idea of the gene expression within the fibrotic environment. In the second approach, elutriation-enriched (>95%) carcinoid tumor cells from the three different groups were examined. These cells were obtained from the same material as that used for the whole tissue sections. The minimum number of samples in a group required to give a representative statistical result is three. Histologically identified tumor material was used to provide material for the whole tissue approach.

Small bowel carcinoid and gastric carcinoid tumor cells were isolated and enriched by Dr. Kidd using a procedure that he had developed, which provides a novel method for producing highly enriched cell populations. After I collected fresh tumor tissue in the operating room, I placed part of the specimen in warm RPMI media and delivered it

immediately to Dr. Kidd, who hand dissected the tumor and digested it in collagenase (0.25mg/ml)/DNAse (100U/mL) solution for 60 min at 37°C under constant aeration. A total of 1-2x10^7 tumor cells were obtained from each preparation. Cells were then loaded onto an elutriator at 20ml/min for 3 min at 2,000 rpm, washed for 10 min at 25ml/min at 2,500 rpm, and the fraction of interest was separated by counterflow-elutriation using a flow rate of 55ml/min at a centrifugation speed of 2,000 rpm. This fraction was then applied to a step Nycodenz gradient and centrifuged at 1,100 rpm for 8 min. Tumor cells collect at the interface of a density of 1.070g/L, and the final cell count was 1-2x10^6 cells. This provided sufficient RNA (4μg) for both GeneChip® and Q RT-PCR analysis.

RNA isolation (performed by Dr. Kidd): Total RNA was extracted from tumor tissue or from cells using the RNeasy kit for lipid tissue (Qiagen, Valencia, CA.) and the quality assessed by the Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA.) to visually verify the absence of genomic DNA contamination, integrity and ratio of 28S and 18S bands, and by a A_{260}/A_{280} ratio which was at least 1.8.

GeneChip® Technology: Dr. Kidd generated target cRNAs and hybridized to human HU133A using a GeneChip® approach (Affymetrix, Santa Clara, CA). The intensity was equally scaled for each chip (intensity = 500) and genes normalized using the RMA algorithm. Thereafter, differences in gene expression in the different groups were examined using GeneSpring software.

The Affymetrix U133 Plus 2.0 array consists of over 1,300,000 unique oligonucleotide sequences, which represent over 47,000 transcripts. The hybridized arrays were scanned using a GeneChip® scanner 3000 (Affymetrix Inc. CA) at the Affymetrix

Resource facility at Yale University. Arrays were scaled to an average intensity of 500 and analyzed independently using Microarray Suite (MAS) 5.0 software (Affymetrix, Santa Clara, CA). The hybridization intensity data will be converted into presence/absence calls for each gene, and changes in gene expression between experiments were detected by comparison analysis. The data was further analyzed by Dr. Kidd using NetAffx® (Affymetrix, Santa Clara, CA) and Gene Spring® (Silicon Genetics, Redwood City, CA.).

In the first set of experiments, tumors from gastric carcinoid patients were used as a baseline for the comparison with the two groups of small bowel carcinoid tumors (invasive, non-fibrotic tumors and fibrotic tumors). Each candidate gene was normalized to the median/mean intensity of that gene in the gastric carcinoid tissue. Only those genes with a minimum signal intensity of 200 and where the average fold change of the small bowel carcinoid patients was at least 1.5 were further analyzed. The Student one sample *t*-test, with a p-value cut off of 0.05, was performed by Dr. Kidd to determine if the average ratio of the small bowel carcinoid tumors was significantly different from 1.0, which would be the value of the gastric carcinoid samples after normalization. Benjamini and Hochberg false discovery rate was used for multiple testing corrections[225]. Fibrosis-associated genes were defined as those significantly up-regulated in the fibrotic small bowel carcinoid group but not in either the non-fibrotic small bowel carcinoid or non-fibrotic gastric carcinoid groups. In the second set of experiments, fibrotic small bowel carcinoids were examined against the baseline of normal small bowel mucosa. Only those genes identified to be over-expressed in both sets of experiments were examined further.

**Summary statement of my specific role in the design and completion of this study:*

I recruited all of the patients who participated in our study at Dr. Modlin's weekly neuroendocrine tumor clinic, and I occasionally attended Dr. Murren's oncology clinic to recruit additional patients. I was in regular contact with Marianne Davies, Jan Napoletano, and Marianne Gallipoli, all nurses at the oncology clinic, who notified me when new patients presented to clinic with neuroendocrine tumors. I consented every new patient for the study, performed the phlebotomy to obtain serum samples, and I centrifuged each sample after collection and isolated the serum by myself as described, which I then stored in the lab freezer. I also interviewed each patient at clinic to obtain their medical history and collected further information by reviewing their charts retrospectively. I collaborated with Dr. Vitali Khomitch in the Department of Pathology, and he informed me whenever surgical pathology identified a carcinoid tumor in a specimen. If the patient was still in-house, Dr. Khomitch notified me so that I could recruit the patient for our study and collect a serum sample.

For tissue procurement, I regularly checked the operating room schedule to ascertain whether any cases were scheduled in which a tissue sample might become available, and several of the general surgeons at YNHH notified me in advance when performing resection of gastrointestinal tumors. When tissue was made available, I was present in the operating room to collect the specimen, dissect the tissue of interest (e.g. tumor, adjacent normal mucosa, etc.), and I then stored it immediately in the -80°C freezer and/or in warm RPMI media, the latter of which I would deliver to Dr. Kidd, the director of our lab, for his cell culture experiments. The comprehensive tissue analysis included specimens that had been collected prior to my joining the lab, but I collected every sample that became available throughout the course of this project. Because this study was conducted over the course of a year, I was able to follow several of our patients closely over several months to determine the

objectives detailed in part 6 of the "Results" chapter ("effects of medical or surgical intervention on CTGF serum levels"). Among the patients who presented for surgical resection of bowel tumors, I performed daily serial phlebotomies post-operatively in addition to the pre-operative sample I collected.

The RNA isolation and RT-PCR experiments were performed exclusively by Dr. Kidd, and the carcinoid tissue microarray (TMA) had been constructed by Drs. Kidd and Camp prior to the start of this project. However, the tissue I collected over the course of the study was used to construct a second TMA that paired carcinoid tumor tissue with adjacent normal mucosa. Immunohistochemical staining and AQUA analysis using the microarray was however performed jointly by Dr. Kidd and me. We did the staining experiments together and prepared the multi-color staining images using Photoshop. We performed the AQUA analysis in Dr. Camp's lab at Yale using the protocol described. In addition, we contributed equally to the data analysis by using Microsoft Excel for statistical analysis.

I performed all serum ELISA assays with the assistance of Dr. Kidd, and we used commercially available kits for each protein as described. We used a microplate reader in a lab on the first floor of the BML research building to analyze the wells, and the protein concentrations were calculated according to the guidelines outlined in the protocol of each ELISA kit described and computation analysis was facilitated by using Microsoft Excel. I performed all statistical analysis of the ELISA data with Dr. Kidd's guidance and used this data to construct the bar graphs that were included in the experimental section.

The GeneChip® analysis was completed by the Affymetrix Resource facility at Yale, and while the samples I collected were used in the analysis, the experiments (e.g. RNA isolation) were performed by Dr. Kidd. We collaborated on the biostatistical analysis once the

data from these experiments were made available and worked together to prepare the figures illustrating the data in my thesis.

Of note, the data presented in the thesis, as well as several of the figures, have been presented at several meetings over the past year, including a surgical research seminar at Yale in April of 2004, Student Research Day in May 2004, and at poster sessions at the January 2004 ASCO meeting in San Francisco and at the May 2004 DDW conference in New Orleans. My preliminary data were included in my October 2003 Ohse grant application, and Dr. Kidd used some of the data we generated in his recent Ohse grant application as well. Dr. Modlin used some of my collages as part of his Grand Rounds presentation in October 2004.

RESULTS

1. EVIDENCE FOR CTGF EXPRESSION IN SMALL BOWEL CARCINOID TUMORS AND NORMAL SMALL BOWEL MUCOSA

a) CTGF expression in normal small bowel tissue:

Immunofluorescent localization of CTGF in normal small bowel mucosa (*Figure 23*) and small bowel carcinoid tumors (*Figure 24*) was performed in paraffin sections. CTGF was localized in the cytoplasm of cells. In the normal small bowel, CTGF-positive cells were identified in the base of the crypts. A dual staining approach identified low numbers of double-stained CTGF and CgA- (15±5%) or serotonin-positive (30±12%) cells (*Figure 23*).

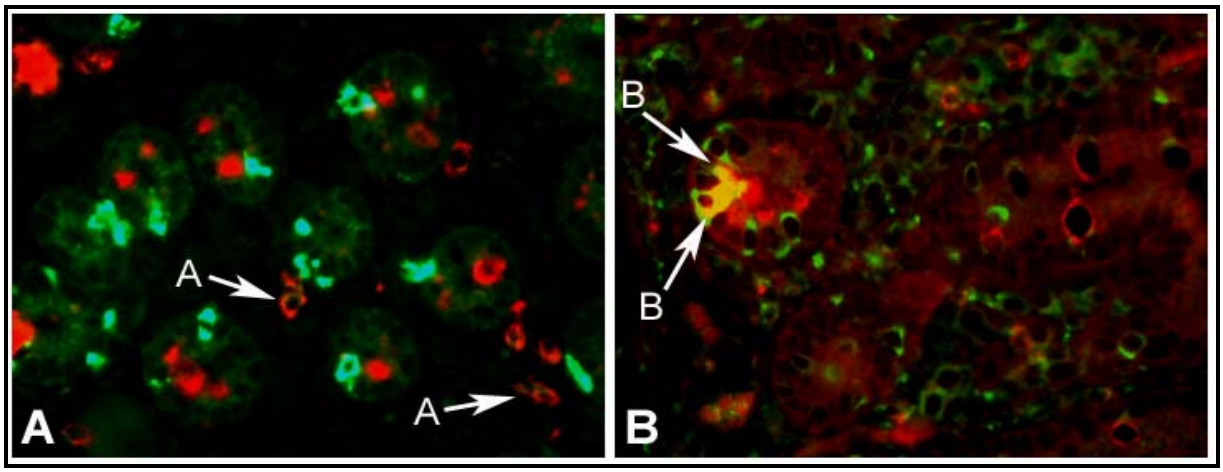


***Figure 23A**: Dual color staining of CgA (green – Alexa 488) and CTGF (red – Cy5) in normal small intestinal mucosa (cross-section). Dual-stained cells are indicated by arrows (A). A minority of CgA cells (~15%) was also CTGF positive (400x magnification).*

***Figure 23B**: Dual color staining of CTGF (red – Cy5) and serotonin (green – Alexa 488) in normal small intestinal mucosa (cross-section). Dual-stained cells (red + green = yellow) are indicated by arrows (B). A minority of serotonin-producing cells (~30%) was also CTGF positive (600x magnification).*

b) CTGF expression in small bowel carcinoid tumors:

In the small bowel carcinoid tumors, co-staining with anti-CgA (*Figure 24A*) or anti-serotonin (*Figure 24B*) antibodies demonstrated a significant co-localization with CTGF and

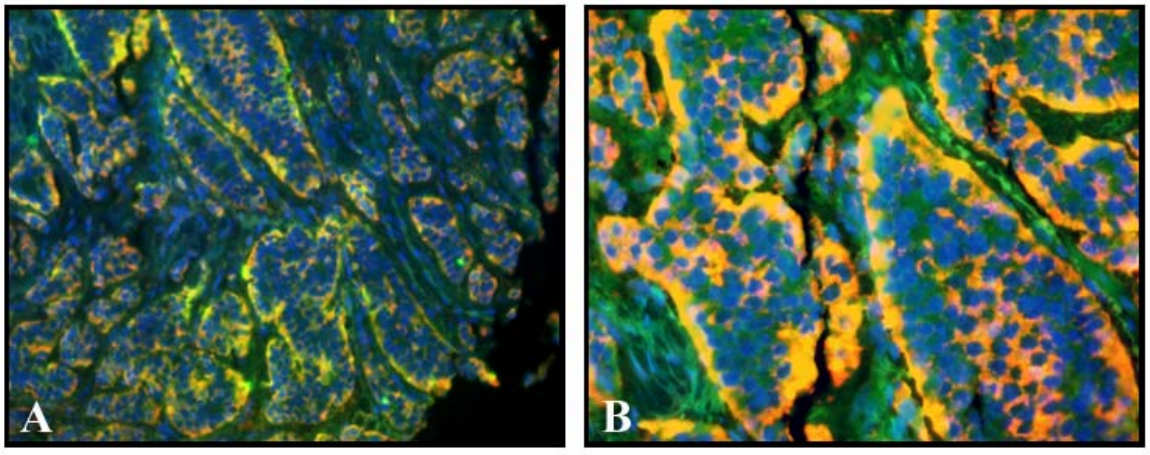


Figure 24A*: Triple color staining of nuclei (blue – DAPI), CgA (green – Alexa 488) and CTGF (red – Cy5) in a carcinoid tumor from the carcinoid tissue microarray. Staining for both CgA and CTGF was cytoplasmic. Dual-stained (CgA + CTGF) cells are yellow. A majority of CgA cells (~80%) were also CTGF positive. (400x magnification).*

Figure 24B*: Triple color staining of nuclei (blue – DAPI), serotonin (green – Alexa 488) and CTGF (red – Cy5) in a carcinoid tumor from the carcinoid tissue microarray. Staining for both Serotonin and CTGF was cytoplasmic. Dual-stained (Serotonin + CTGF) cells are yellow. A majority of the serotonin cells (~95%) were also CTGF positive. (600x magnification).*

either antibody (80±12% and 93±6% respectively) in tumor mucosa. These results demonstrate that the majority of small bowel carcinoid tumor cells express CTGF (90%).

c) The CTGF secretory pathway in small bowel tumor cells:

Serial sections from carcinoid tumors were stained for nuclei and CTGF or the specific Golgi-marker protein 58K. Staining demonstrated overlapping distribution of CTGF and 58K, indicating that CTGF was synthesized via the constitutive pathway in small bowel carcinoid tumor cells (*Figure 25*). This is consistent with the findings in dermal fibroblasts and activated hepatic stellate cells[226]. These results demonstrate that CTGF is actively synthesized in the Golgi network in the tumor cells.

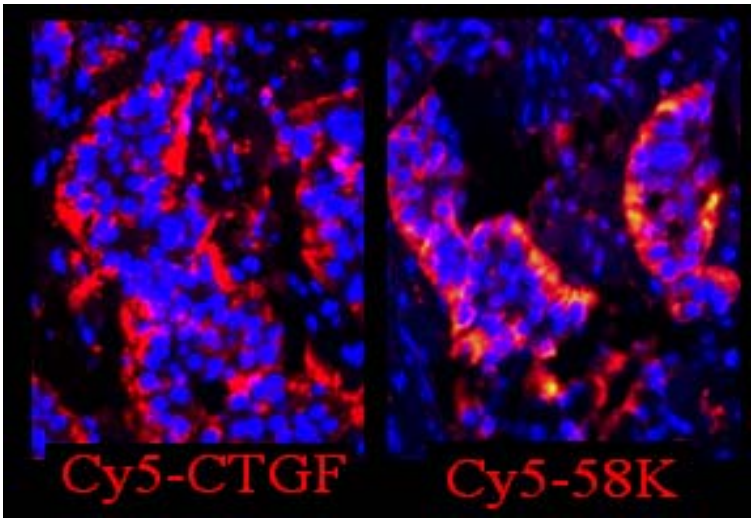


Figure 25: *Dual color staining of nuclei (blue – DAPI) and either CTGF (red – Cy5) [left] or anti-58K (golgi staining) (red – Cy5) [right] in 2 serial sections from the same small bowel carcinoid tumor. These show overlapping distribution of CTGF and the Golgi apparatus demonstrating that CTGF is secreted through the constitutive pathway in the tumor.*

2. QUANTITATIVE RT-PCR ANALYSIS DEMONSTRATES CTGF AND TGFβ1 EXPRESSION IN SMALL BOWEL AND GASTRIC CARCINOID TUMORS

Quantitative RT-PCR (Q RT-PCR) analysis was undertaken using Assays on Demand (Applied Biosystems) on the RNA isolated from small bowel EC carcinoids (*n=5*); gastric ECL cell tumors (*n=5*); normal small bowel samples (*n=4*) and normal gastric mucosa (*n=5*) to quantitatively measure the levels of CTGF and TGFβ1 mRNA expression in these two different tumor types. Small bowel tumors develop fibrosis while gastric carcinoids do not.

a) Small bowel versus gastric carcinoids:

Message for both CTGF and TGFβ1 were significantly elevated in the 5 small bowel carcinoid tumor samples ($p<0.05$ vs. normal mucosa) (*Figure 26*). In contrast, although TGFβ1 message was not different (+1.13–fold) in gastric carcinoid tumor samples, message

levels of CTGF were significantly decreased (-1.3–fold; $p<0.01$) compared to small bowel carcinoid tumors (*Figure 26*). These results demonstrate that both gastric and small bowel carcinoids express mRNA for TGFβ1, and that CTGF message is over-expressed only in small bowel carcinoid tumors.

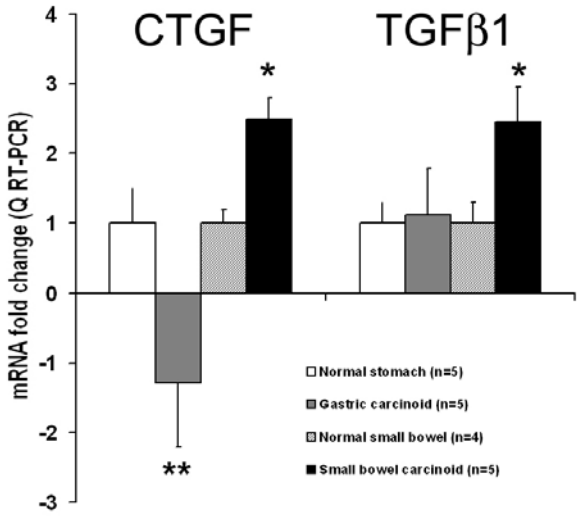


***Figure 26**: Message levels of both CTGF and TGFβ1 determined by Q RT-PCR. Levels were corrected against expression of the housekeeping gene, GAPDH, compared to similarly corrected gene levels in normal mucosa, and represented as fold increase over normal (1.0). CTGF and TGFβ1 were significantly over-expressed (~2.5-fold) in small bowel carcinoid tumor samples compared to normal mucosa (*p<0.05) but not the gastric carcinoids. Gastric carcinoids had significantly decreased CTGF compared to small bowel carcinoid tumors (**p<0.01). Mean±SEM, n=4-5.*

b) Correlation between CTGF and TGFβ1:

There was a good correlation ($R^2=0.95$) between CTGF and TGFβ1 message levels (*Figure 27*) in the small bowel carcinoid samples demonstrating that transcription of these growth factors was tightly associated in this tumor tissue. These results thus demonstrate that CTGF and TGFβ1 message levels are strongly correlated.

3. A FUNCTIONAL TGFβ1/CTGF AXIS SPECIFICALLY OCCURS IN SMALL BOWEL TUMORS ASSOCIATED WITH CLINICALLY DOCUMENTED PERITONEAL FIBROSIS

The immunohistochemical expression of the TGFβ1 pathway in gastric carcinoids and in small bowel carcinoid tumors from patients with or without clinically relevant retroperitoneal

fibrosis was examined (*Figure 28*).

a) TGFβ1: Initially, the expression of TGFβ1—the upstream effector of CTGF synthesis—was examined in order to assess whether expression levels of this growth factor could differentiate patients with or without fibrosis. We noted that patients with gastric carcinoids had high TGFβ1 scores (AQUA score: 93.7±5). Although TGFβ1 expression levels were elevated in patients with fibrosis (AQUA score: 90.6±4.4) compared to the patients with no evidence of fibrotic disease (AQUA score: 82.7±4) this was not statistically different (p=0.12).

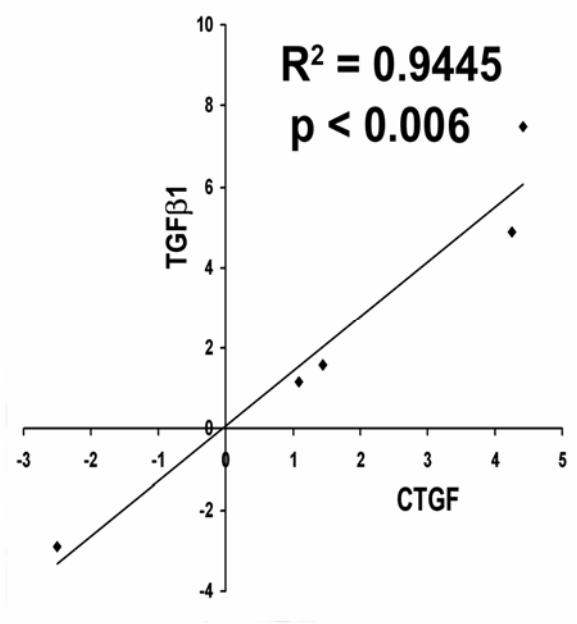


***Figure 27**: Correlation analysis of Q RT-PCR results in small bowel EC carcinoids. There was a good correlation between CTGF and TGFβ1 message levels in tumor samples (R^2=0.95, p<0.006, n=5).*

b) TGFβRII: TGFβ1 mediates its effects by binding and activating the membrane receptor – TGFβRII. We investigated staining of this receptor to test whether this was altered during fibrosis.

Significantly higher scores for TGFβRII were noted in patients with fibrosis (AQUA Score: 36.2±4.5) compared to patients without fibrosis (AQUA Score: 22.3±2.5; p<0.01). Gastric carcinoid patients had an intermediate score (29.3±2.3). This indicates that TGFβRII may be down-regulated in small bowel carcinoid tumor patients that do not generate fibrosis. Alternatively, the receptor may be over-expressed on tumor cells.

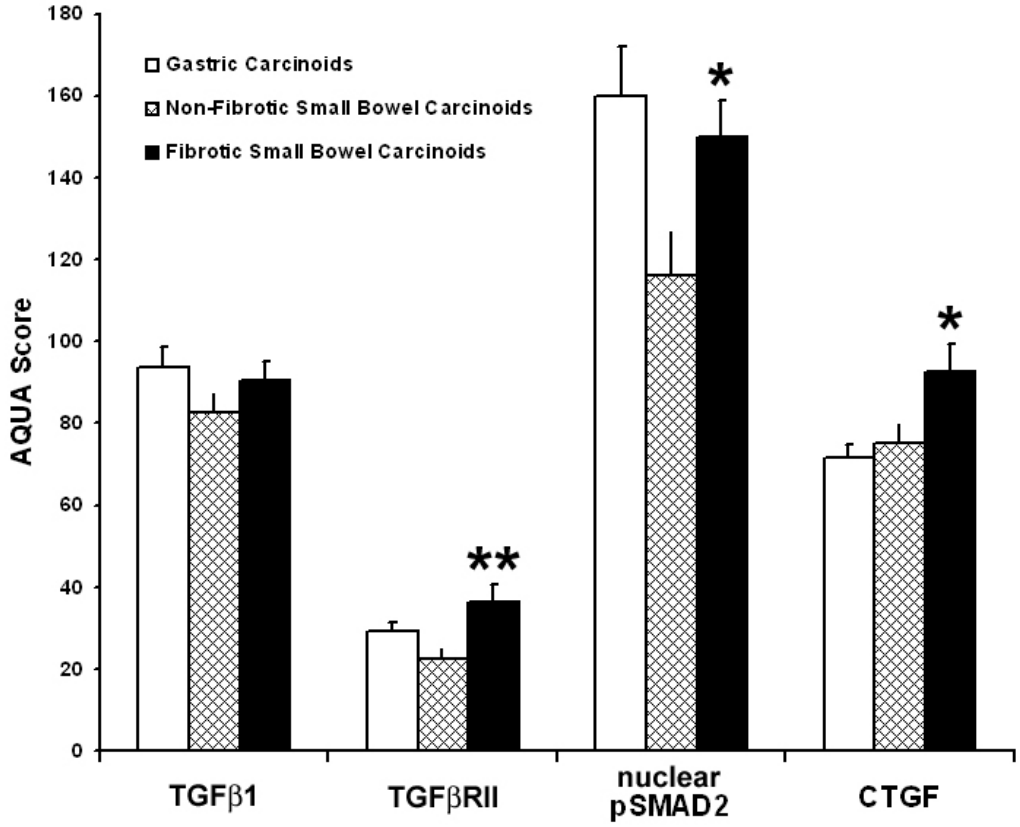


Figure 28*: AQUA scores for TGFβ1 pathway protein expression in patients with small bowel carcinoids (with or without clinically documented fibrosis) or gastric carcinoids on the tissue microarray. Levels of TGFβ1 were not different. TGFβ1RII levels were increased in patients with fibrosis compared to non-fibrotic patients (p<0.01). Receptor levels were also elevated in gastric carcinoid patients but this was not significant. Nuclear staining of phosphorylated SMAD2 was elevated (p<0.03) in fibrotic compared to non-fibrotic patients. Levels were also significantly elevated in gastric carcinoid tumors (p<0.05) compared to non-fibrotic patients. CTGF, the downstream target of the TGFβ pathway was significantly increased only in patients with fibrosis. (*p<0.05, **p<0.01). Mean±SEM, n=15-22.*

Mean immunostaining levels for non-carcinoid tissue (liver, bowel tissue) were:

	TGFβ1	TGFβRII	pSMAD	CTGF
Liver	60±3	12±2	85±7	61±5
Bowel	52±5	9±2	74±6	70±12

c) *Phosphorylated SMAD2:* Activation of TGFβRII following TGFβ1 binding results in phosphorylation of the SMAD intracellular signaling pathway. Specifically, SMAD2 is phosphorylated and translocates to the nucleus where it is involved in mediating

transcription.

High AQUA scores of nuclear pSMAD2 were noted in gastric carcinoids (160±12), indicating that these cells are TGFβ1 responsive. Higher pSMAD2 AQUA scores were noted in patients with fibrosis (150±9) compared to those without fibrosis (116±11; $p<0.05$). This suggests that both gastric and small bowel carcinoid tumors (associated with fibrosis) demonstrate a functional response to TGFβ1.

d) CTGF: CTGF is the downstream target of TGFβ1 signaling. TGFβ1 specifically initiates transcription of the CTGF gene in cells that are responsive to this pathway. Higher levels of CTGF staining (AQUA score: 92.5±8.2; p=0.017) were identified in small bowel carcinoid patients with fibrosis compared to the patients (AQUA score: 72.7±3.2) with no clinical evidence of fibrotic disease. In addition, despite a functional TGFβ1 pathway, CTGF was not highly expressed in the gastric carcinoids.

4. SERUM LEVELS OF CTGF AND TGFβ1 ARE ELEVATED IN PATIENTS WITH SMALL BOWEL AND GASTRIC CARCINOID TUMORS

Having established that CTGF message and protein levels were elevated in patients with small bowel carcinoids, we next evaluated whether serum levels of CTGF were measurable and if they correlated with tissue levels. In addition, we examined serum levels of TGFβ1, and the two carcinoid secretory products Chromogranin A and serotonin. The latter were included because these are carcinoid tumor markers and serotonin has been proposed as a potential marker of cardiac fibrosis[90].

a) CTGF and TGFβ1 in normal subjects and in carcinoid patients: Serum levels of

CTGF ranged from 7.2–60 ng/mL and TGFβ1 from 5.6–88 ng/mL. Significantly higher serum CTGF levels were found in patients with small bowel carcinoids (24.2±1.8) than in patients with gastric carcinoids (11.8±0.7, $p<0.03$) and normal subjects (10±0.2, $p<0.02$) (*Figure 29*). Serum levels of TGFβ1 were also elevated in patients with small bowel carcinoids (35.4±1.4) compared to gastric carcinoid patients (22.5±1.2, $p<0.02$) and normal subjects (23.9±5, $p<0.04$). Comparing serum CTGF levels with tissue levels (AQUA scores) identified a strong correlation between these two measurements ($R^2=0.91$, $p<0.005$, $n=9$).

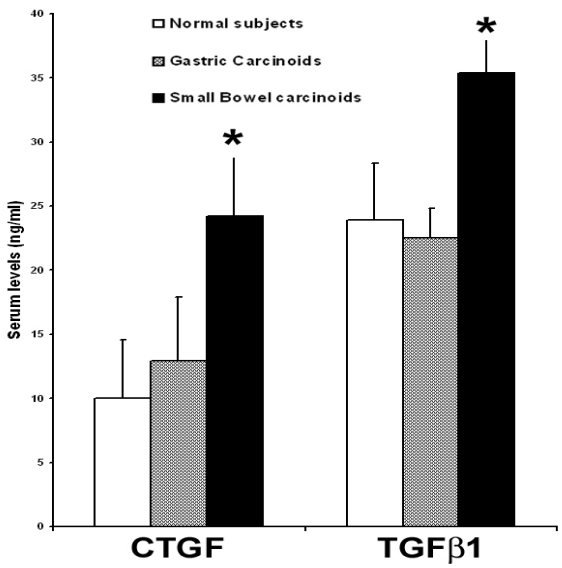


***Figure 29**: Serum levels of CTGF and TGFβ1 in normal subjects, patients with gastric carcinoids and patients with small bowel carcinoids. Levels (ng/mL) of CTGF were significantly elevated (>2-fold) in patients with small bowel carcinoids compared to normal subjects or patients with gastric carcinoids. Levels (ng/mL) of TGFβ1 were elevated ~1.5-fold in small bowel carcinoid patients compared to gastric carcinoids and versus normal subjects. *p<0.02 vs. normal subjects and gastric carcinoids. Mean±SEM. n=10-12.*

b) *Chromogranin A and serotonin in normal subjects and in carcinoid patients:* Serum levels of Chromogranin A ranged from 2–976 U/L and serotonin from 9–1247 ng/ml. Gastric (89±16, $p<0.05$) and small bowel carcinoid (298±111, $p<0.01$) patients had significantly elevated Chromogranin A levels compared to normal subjects (14±7) (Figure 30). Small bowel carcinoid patients had elevated serotonin levels (250±52, $p<0.01$) compared to gastric carcinoid patients and normal subjects.

These two markers are important for several reasons: they distinguish carcinoid tumors; serum serotonin is a marker for EC cells; and serum levels of serotonin have been shown to be related to cardiac fibrosis.

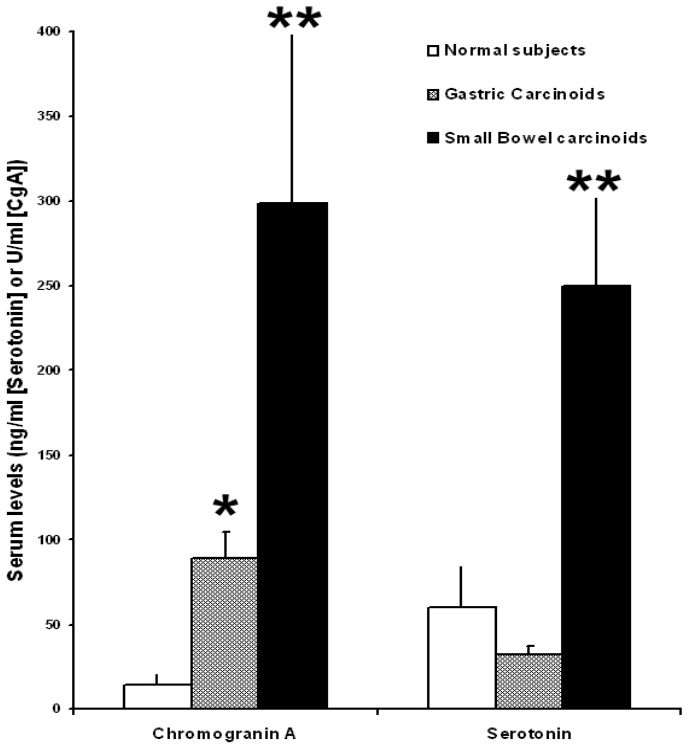


***Figure 30**: Serum levels of Chromogranin A and serotonin in normal subjects, patients with gastric carcinoids and patients with small bowel carcinoids. Levels (U/mL) of Chromogranin A were significantly elevated in patients with gastric and small bowel carcinoids compared to normal subjects. Levels (ng/mL) of serotonin were elevated in small bowel carcinoid patients compared to gastric carcinoids and versus normal subjects. *p<0.05 vs. normals, **p<0.01 vs. normals and gastric carcinoids. Mean±SEM. n=10-*

Collectively, these results demonstrate that serum levels of CTGF and TGFβ1 are elevated in small bowel carcinoid patients compared to those with gastric carcinoids, and that serum and tissue levels of CTGF are highly correlated. Furthermore, small bowel carcinoid tumors secrete Chromogranin A and serotonin at detectable levels as expected.

5. SENSITIVITY AND SPECIFICITY OF CTGF SERUM LEVELS IN PATIENTS WITH SMALL BOWEL CARCINOID TUMORS

Calculations of sensitivity and specificity of CTGF were undertaken on CTGF levels from 30 patients (small bowel carcinoids [*n=10*], gastric carcinoids [10], normal subjects [10]). CTGF levels were set at 10 ng/ml (upper level of normal) and 15ng/ml (upper level

identified in gastric carcinoid patients). The sensitivity and specificity for detecting patients with small bowel carcinoids versus gastric carcinoids or normal subjects is shown in **Table 1**.

Table 1. Sensitivity and specificity of CTGF as a biomarker

	Small bowel vs. Normal		Gastric vs. Normal		Small bowel vs. Gastric	
CTGF cut-off level	10ng/ml	15ng/ml	10ng/ml	15ng/ml	10ng/ml	15ng/ml
Sensitivity (%)	100	70	70	0	100	70
Specificity (%)	100	100	100	100	30	100

Three of 10 small bowel carcinoid patients had symptomatology consistent with disseminated disease; the serum CTGF levels in these patients were elevated and ranged from 37-60 ng/ml. These results demonstrate that serum CTGF levels specifically identify patients with small bowel carcinoids with a sensitivity that is dependent on patient symptomatology.

6. EFFECTS OF MEDICAL OR SURGICAL INTERVENTION ON CTGF SERUM LEVELS IN PATIENTS WITH SMALL BOWEL CARCINOID TUMORS

Having established that CTGF was secreted and detectable in the serum of patients with small bowel carcinoid tumors, the next objective was to evaluate how consistent the levels of this growth factor were. I examined 6 patients at 3-month intervals to assess the inter-patient variability. I next examined the effects of medical intervention (e.g. octreotide therapy) or surgical intervention on serum levels of this growth factor.

a) Inter-patient variability: Six patients with gastric ($n=3$) or small bowel ($n=3$) carcinoid tumors were examined 3 months apart. Initial serum levels ranged from 10-60 ng/ml and at 3 months ranged between 10-58 ng/ml. The mean serum level at entrance was 21.8±7.8 and at 3 months was 20±7.8. A 2-tailed paired *t*-test identified no difference

between the two sample sets (p=0.40). The coefficient of reproducibility of the two samples was 95.5% (*Figure 31*).

b) Medical intervention: Four patients who were treated with long-acting somatostatin analogs were examined before and 24 hours after somatostatin analog injection. No significant differences were noted (28±9 vs. 31±12; p=0.4).

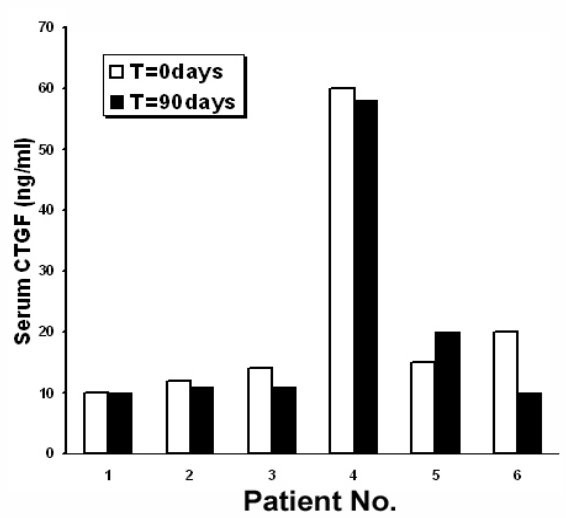


Figure 31*: Temporal relationship of serum CTGF levels in gastric and small bowel carcinoid patients. There was a good temporal correlation for this growth factor (Coefficient of reproducibility >95%, n=6).*

c) Surgical intervention: Serum levels of CTGF were examined in four carcinoid patients who underwent surgery for removal of their tumors. Serum was collected pre-operatively and 24 hours post-operatively. No appreciable differences were noted in CTGF levels post-operatively (15.2±2 vs. 12±1.2; p=0.31) (*Figure 32*). All four patients had extensive mesenteric tumor and hepatic metastases. These results demonstrate that CTGF levels remain elevated because hepatic metastases still produce CTGF (*Figure 33*). Patients with extensive disease would be predicted to develop fibrosis, an outcome consistent with the hypothesis.

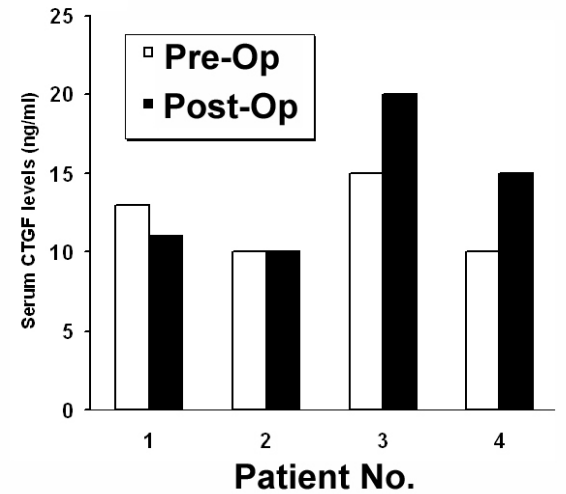


Figure 32*: CTGF serum levels in four carcinoid patients before (pre-operatively) and one-day post-operatively. CTGF levels did not appreciably change (n=4).*

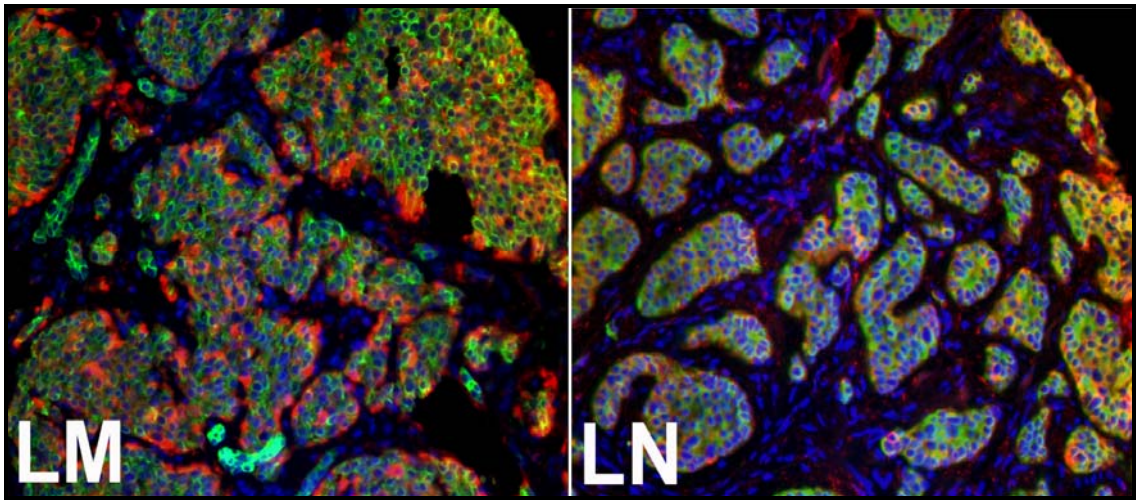


***Figure 33**: Triple color staining of nuclei (blue – DAPI), cytokeratin (green – Alexa 488) and CTGF (red – Cy5) in liver metastases (LM) and lymph node (LN) material. Extensive CTGF staining is present in all tumor cells (400x magnification). AQUA scores for CTGF in liver metastases (95±12) and lymph node material (87±13) were similar to primary tumor levels (92±9) and significantly elevated ($p<0.01$) versus normal mucosa (62±5).*

These results suggest that serum levels of CTGF remain stable over a 3-month period; surgical intervention did not significantly alter serum CTGF levels after 24 hours; and liver and lymph node metastases express CTGF.

7. GeneChip® Analysis of Gastric and Small Bowel Carcinoids

Material from 29 tissue blocks (*n=23* patients) was examined using Affymetrix GeneChip® analysis. Total RNA was isolated from each sample using the RNeasy kit (Qiagen). Target cRNAs were generated and hybridized to human HU133A GeneChip® (Affymetrix, Santa Clara, CA). The intensity was equally scaled for each chip (intensity = 500) and genes normalized using the RMA algorithm. Thereafter, differences in gene expression in the different groups were examined using GeneSpring® software.

a) Cluster analysis of gene expression: Global gene expression patterns clearly distinguished between the two different organs (stomach and small bowel) as well as the different tumor types (gastric and invasive small bowel carcinoid) (*Figure 34*).

b) GeneSpring analysis: GeneSpring analysis identified 1294 gene significantly altered in the non-fibrotic gastric carcinoid tumors and 1709 genes in the invasive small bowel carcinoids compared to normal gastric and small bowel mucosa respectively. Direct comparison of gene expression in each tumor group identified 1294 genes that significantly altered between them; 375 genes were up-regulated in small bowel carcinoids and 919 down-regulated.

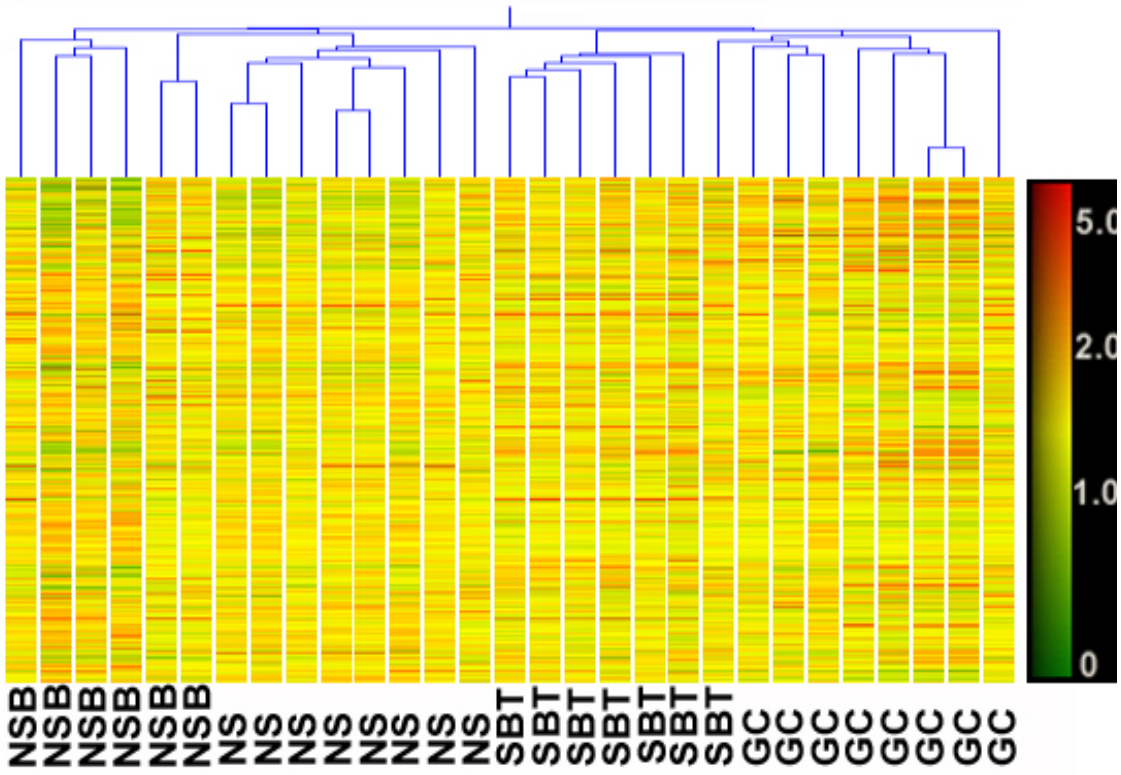


Figure 34*: Hierarchical clustering of 29 Affymetrix chips including normal stomach (NS; n=8), gastric carcinoid (GC; n=8), normal small bowel (NSB; n=6) and small bowel carcinoid tumors (SBC; n=7). Expression levels 0-5.0 are indicated on the right.*

c) Fibrotic genes specifically expressed in small bowel carcinoids compared to gastric carcinoids: The gene profiles of these two tumor types were next examined to identify whether fibrosis-associated genes were selectively expressed in small bowel carcinoids. Specifically, the genes identified to be useful markers in liver and pancreatic fibrosis were examined. These genes included hyaline genes, YKL-40, gamma-glutamyl transpeptidase (GGT), alpha-2-macroglobulin, procollagen type III N-terminal peptide (PIII-NP), and the matrix metalloproteinases (MMP-1, MMP-2, MMP-9)[213-220]. Genes that were significantly altered in invasive small carcinoids were tabulated (*Table 2*).

Table 2. Potentially clinically relevant fibrotic associated genes in invasive small bowel carcinoids.

Small bowel carcinoid tumor versus Gastric carcinoid			**Small bowel carcinoid tumor versus Normal small bowel**		
Gene	Fold-change	*p-value*	Gene	Fold-change	*p-value*
apolipoprotein A-I	+13.7	0.0054	apolipoprotein A-I	NS	NS
apolipoprotein A-I	+12.05	0.011	apolipoprotein A-I	NS	NS
α-2-macroglobulin	**+1.97**	**0.034**	**α-2-macroglobulin**	**+2.32**	**0.005**
procollagen (type III) N-endopeptidase	+1.52	0.011	procollagen (type III) N-endopeptidase	NS	NS

Although apolipoprotein A-I and procollagen N-endopeptidase were over-expressed in small bowel carcinoids, they were not different when examined against normal small bowel mucosa.

These results indicate that mRNA for alpha-2 macroglobulin, whose protein product has been demonstrated to have a sensitivity and specificity of 75% and 67%, respectively, for predicting significant fibrosis in patients with liver disease[227], is upregulated in invasive small

bowel carcinoids. Alpha-2 macroglobulin shares a common receptor with CTGF. An examination of CTGF demonstrated that this gene was variably expressed in small bowel carcinoid samples (+0.4→+3.5-fold). This supports the hypothesis that CTGF is a selectively expressed gene in small bowel carcinoids.

DISCUSSION

The consequences of peritoneal and cardiac fibrosis are major clinical issues associated with carcinoid tumors of the small bowel. Because so little is understood of the mechanistic basis of fibrosis, there is no method to detect it before it causes either bowel obstruction or cardiac valve dysfunction, nor is there a treatment to control carcinoid-related fibrosis. As opposed to healing tissue, carcinoid tumors provide a unique, homogenous, well-defined cell system to study the evolution of fibrosis.

An interdisciplinary approach using clinical material, DNA microarrays, protein expression profiling and tissue microarray analysis has allowed us to identify CTGF as the most likely mediator associated with small bowel carcinoid fibrosis. The proteomic and tissue microarray analysis in these studies demonstrated that at a tissue level small bowel carcinoids, like gastric carcinoids, have an intact, active TGFβ1 signaling pathway, but only the small bowel carcinoids synthesize and secrete CTGF at clinically significant elevated levels compared to both the normal small bowel mucosa and the non-fibrotic ECL cell carcinoid tumors. Since only the EC carcinoids are associated with fibrosis, our results support the role of CTGF as a candidate regulator of small bowel carcinoid fibrosis. Serum levels of both CTGF and TGFβ1 were significantly increased in ileal carcinoid tumors compared to other GI neuroendocrine tumors, and the results demonstrated strong correlations between serum CTGF and TGFβ1 and between CTGF and CgA, indicating firstly that CTGF and TGFβ1 are co-secreted, and that CTGF may be not only a marker for fibrosis but in addition a marker specific to ileal EC carcinoid tumors.

Our data further demonstrates that small bowel carcinoids have >2-fold levels of CTGF compared to gastric carcinoids, and patients with symptomatology consistent with metastatic disease have >5-fold levels of CTGF. Elevated levels of CTGF (>14ng/ml) are highly correlated with fibrosis and identify scleroderma patients[11], and 70% of midgut carcinoid patients have CTGF levels >15ng/ml. Up to 50% of these patients will develop local or distant (cardiac) fibrosis. Furthermore, we were able to demonstrate that serum CTGF levels in patients with carcinoids are stable over a 3-month period and therefore monitoring this protein has predictive utility; that long-acting somatostatin analogue therapy (octreotide) does not significantly alter serum CTGF levels; and that surgery only causes a transient increase in CTGF that returns to pre-operative baseline within 24 hours. These results suggest that CTGF can identify small bowel carcinoid patients and potentially has sufficient discrimination for the detection of fibrosis.

Additionally, we were able to identify, using the GeneChip® studies in normal gastric and small bowel mucosa and gastric and small bowel carcinoids (n=29 samples), that the candidate fibrotic factor, alpha-2-macroglobulin (which shares a common receptor with CTGF) is over-expressed in invasive midgut carcinoids. This has been shown to be relevant in hepatic stellate cell-driven fibrosis and may provide an alternative factor to examine in small bowel carcinoid fibrosis.

A molecular understanding of carcinoid development and progression is an important step toward the identification of biomarkers with increased specificity for fibrosis and for the development of novel fibrosis-specific therapeutic targets. While TGFβ has long been a therapeutic target for the treatment of fibrotic disorders, it also has normal functions in the body that make chronic administration of any inhibitor that indiscriminately blocks TGFβ

activity problematic due to unwanted side effects. Selectively inhibiting CTGF activity in the body would be a better strategy, as its unregulated over-expression is what leads to persistent, chronic fibrosis. The use of antibodies that inhibit CTGF has the potential to arrest the progression of fibrosis and provide an opportunity to preempt the local and systemic complications commonly associated with small bowel carcinoid tumors. It is also probable that such an approach in these tumors will be applicable to other fibrotic disease processes as well.

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